Study of the cytotoxicity of raw materials of cosmetic and topical pharmaceutical formulations

Verónica Patrícia Moreira da Rocha
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Departamento de Química e Bioquímica
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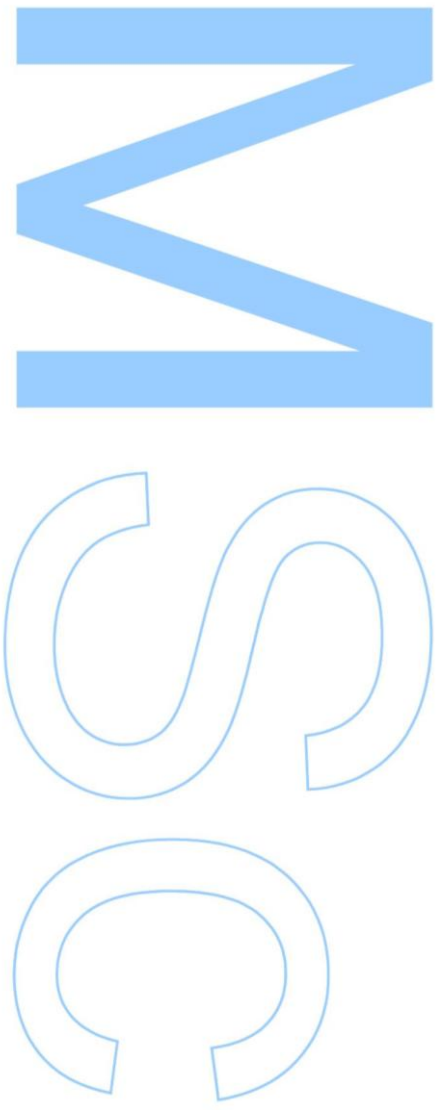
Orientadora
Isabel Almeida, Professora assistente, FFUP
Co-orientadora
Cláudia Marques, Doutorada, FFUP



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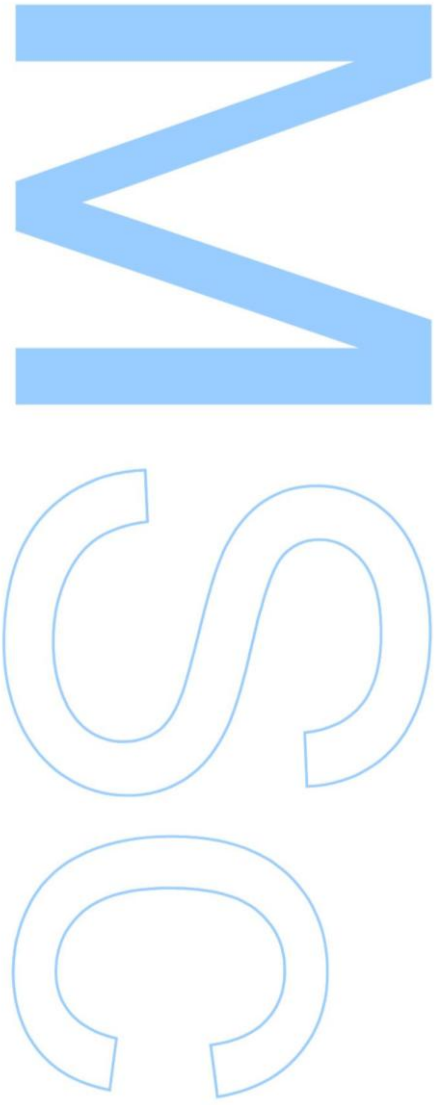

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ABSTRACT

The assessment of the toxicological profile of the raw materials of cosmetics and pharmaceutical formulations is a regulatory requirement. This evaluation should take into account the route of administration. Considering the specific case of topical administration, the preliminary toxicological evaluation of new ingredients can be conducted in skin cell lines, in particular keratinocytes and fibroblasts. The objective of this work was to study the cytotoxicity of ingredients with potential interest for skin application in human keratinocytes (HaCaT cell line). The tested compounds included novel substances obtained by chemical synthesis: 1,2-Dihydroxyxanthone (1,2-DHX), ascorbic acid persulfated (ASS), chlorogenic acid persulfated (ACS) and resveratrol glicoside sulfate (RGS) synthesized in Pharmaceutical Chemistry Laboratory, FFUP. Resveratrol (RSV), resveratrol glicoside (RSV-Gli) and a *Castanea sativa* leaf extract were also tested. The study also included nano and micro particles containing RSV. In an initial step solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) were prepared using a homogenization/sonication technique, and polymeric microparticles produced with a piezoelectric atomizing method. The particles were characterized in relation to size using a laser diffraction technique. To obtain the *C. sativa* extract, dry leaves were extracted with a solution of ethanol:water (7:3). The final dry extract was obtained by lyophilization. Cytotoxicity assays were conducted with an immortalized cell line of human keratinocytes (HaCaT). For further characterization of cytotoxicity, different tests were selected, namely: MTT and AlamarBlue® reduction assays (assessment of metabolic activity), neutral red (NR) uptake (evaluation of lysosomal integrity), exclusion of propidium iodide (PI) and trypan blue (assessment of membrane integrity). The results showed that RSV and its derivatives may be considered non toxic in future studies for concentrations up to 100 µM for RSV, 500 µM RSV-Gli and 1000 µM to RGS. The botanical extract and 1,2-DHX can be safely used up to 100 µg/mL and 50 µM, respectively. The lipid nanoparticles prepared and tested in this work were not considered as promising topical ingredients. Polymeric particles showed deposition on the surface of the cell monolayer and aggregation which led to a great variability of the results. The particles present several challenges for toxicological testing. Other toxicological tests should be performed in order to meet regulatory requirements before considering the use of the compounds tested for cosmetic or pharmaceutical products.

Keywords: raw materials, keratinocytes, nanomaterials, cytotoxicity assays

RESUMO

A avaliação do perfil toxicológico das matérias-primas dos produtos cosméticos e das formas farmacêuticas é uma exigência regulamentar. Esta avaliação deve ter em perspetiva a via de administração. No caso particular da administração cutânea, uma preliminar avaliação toxicológica de novos ingredientes deve ser conduzida em linhas celulares cutâneas nomeadamente, queratinócitos e fibroblastos. O objetivo deste trabalho foi o estudo da citotoxicidade de ingredientes com potencial interesse para aplicação na pele em queratinócitos humanos (linha celular HaCaT). Os compostos testados incluíram substâncias inovadoras obtidas por síntese química: 1,2-di-hidroxixantona (1,2-DHX), ácido ascórbico sulfatado (AAS), ácido clorogénico sulfatado (ACS) e o resveratrol glicosilado sulfatado (RGS), sintetizados no laboratório de Química Farmacêutica, FFUP. Resveratrol (RSV), resveratrol glicosilado (RSV-Gli) e um extrato de folhas de *Castanea sativa* também foram estudados. O estudo incluiu também nano e micro partículas contendo RSV. Numa etapa inicial foram preparadas nanopartículas lipídicas sólidas (SLN) e transportadores lipídicos nanoestruturados (NLC) recorrendo a uma técnica de homogeneização/sonicação, e micropartículas poliméricas produzidas com um método de atomização piezoelétrica. As partículas foram caraterizadas relativamente ao tamanho utilizando uma técnica de difração a laser. Para obtenção do extrato de *C. sativa*, as folhas secas foram extraídas com uma solução de etanol:água (7:3). O extrato final seco foi obtido por liofilização. Os ensaios de citotoxicidade foram realizados com uma linha celular imortalizada de queratinócitos humanos (HaCaT). Para uma caracterização mais aprofundada da citotoxicidade foram selecionados diferentes ensaios, nomeadamente: ensaio de redução do MTT e do AlamarBlue® (avaliação da atividade metabólica), incorporação do vermelho neutro (avaliação da integridade lisossomal), exclusão do iodeto de propídeo (PI) e do azul de tripano (avaliação da integridade da membrana plasmática). Os resultados mostraram que o RSV e os seus derivados, podem ser considerados não tóxicos para estudos futuros em concentrações até 100 µM para o RSV, 500 µM para o RSV-Gli e 1000 µM para o RGS. O extrato botânico e a 1,2-DHX podem ser considerados seguros até 100 µg/mL e 50 µM, respetivamente. As nanopartículas lípidas preparadas e avaliadas neste trabalho não mostraram ser um ingrediente promissor para aplicação tópica. As partículas poliméricas depositaram e agregaram sobre a superfície da monocamada de células, o que conduziu a uma grande variabilidade dos resultados. As partículas apresentam vários desafios para testes toxicológicos. Outros testes toxicológicos devem ser realizados de forma a cumprir os

requesitos regulamentares antes de considerar a utilização dos compostos testados em produtos cosméticos ou farmacêuticos.

Palavras-chave: matérias-primas, queratinócitos, nanomateriais, ensaios de citotoxicidade

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ABBREVIATIONS

1,2-DHX	1,2-Dihidroxyxanthone
ACS	Chlorogenic acid persulfate
ADME	Absorption, Distribution, Metabolism and Excretion
AFC	Aminofluorocoumarin
AM	Acetoxymethyl
API	Active pharmaceutical ingredients
ASS	Ascorbic acid persulfate
ATP	Adenosine Triphosphate
BCA	Bicinchoninic acid
C. sativa	*Castanea sativa*
DMEM	Dulbecco's Modified Eagles's Medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
EDTA	EthyleneDiamineTetraacetic Acid
EtOH	Ethanol
FBS	Fetal Bovine serum
FDA	Fluorescein Diacetate
GF-AFC	Glycylphenylalanyl-Aminofluorocoumerin
HPLC	High Performance Liquid Chromatography
HTS	High Throughput Screening
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
LDH	Lactate Dehydrogenase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NLC	Nanostructured Lipid Carriers
NMs	Nanomaterials
NR	Neutral Red
PI	Propidium Iodide
Poly	Polymeric particles
Poly-RSV	RSV loaded polymeric particles
RGS	Resveratrol Glicoside Sulfate
RSV	Resveratrol
RSV-Gli	Resveratrol Glicoside
SCCS	Scientific Committee on Consumer Safety
SLN	Solid Lipid Nanoparticles
SRB	Sulphorhodamine
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
XTT	sodium 3′-[1-phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitrobenzene) sulfonic acid hydrate

1. INTRODUCTION

1.1 Safety assessment of raw materials for cosmetic and topical pharmaceutical formulations

Millions of consumers use cosmetic and pharmaceutical products on a daily basis. The raw materials of cosmetics or pharmaceutical products may be of plant, mineral or animal source or obtained by chemical synthesis. The determination of their safety profile is of great importance not only for new ingredients but also when a well-known and generally recognized as safe ingredient is used in a higher concentration or with a different application [1]. Regulatory requirements diverge between cosmetics and pharmaceutical products.

The term cosmetic has a broad definition, according to the Cosmetics Regulation, cosmetic product “means any substance or mixture intended to be placed in contact with the external parts of the human body (…) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours” and includes skin care products, hair care products, nail and lip care products, and sunscreens [2].

European Regulations regarding the safety of cosmetics were introduced in 1976 [3] by the European Union’s (EU) Cosmetic Directive which has been periodically updated. In 2009, the legislative recast transforms the Cosmetic Directive 76/768/EEC into a Regulation. According to the Cosmetics Regulation (EC) No 1223/2009, which became fully applicable from 11 July 2013, “a cosmetic product made available on the market shall be safe for human health when used under normal or reasonably foreseeable conditions of use”. The most significant changes introduced by the new Cosmetics Regulation include: strengthened safety requirements for cosmetic products (safety report), introduction of the notion of “responsible person”, centralized notification of all cosmetic products placed on the EU market, introduction of reporting of serious undesirable effects and new rules for the use of nanomaterials in cosmetic products [4].

Cosmetics do not require a pre-marketing approval, being the safety of cosmetics and their ingredients the responsibility of the “responsible person” who must ensure that they undergo an expert scientific safety assessment before they are sold. Each cosmetic product is considered as an individual combination of cosmetic substances. It is generally accepted that the safety evaluation can be done by ascertaining the toxicity of its substances [5]. The safety of substances that cause some concern with respect to human health, are evaluated at the Commission level by a scientific committee, presently called the Scientific Committee on Consumer Safety (SCCS). The SCCS evaluates the safety of ingredients such as colorants, preservatives, and UV filters [6].

The cosmetic product safety report should contain the toxicological profile of the substances included in the cosmetic product for all relevant toxicological endpoints, a particular focus on local toxicity evaluation (skin and eye irritation), skin sensitization, and in the case of UV absorption photo-induced toxicity shall be made [2]. It is noteworthy, the ban on animal testing for cosmetic purposes promoted the development of alternative *in vitro* methodologies for safety evaluation.

Pharmaceutical products, also known as medicines, are a fundamental component of both modern and traditional medicine and are defined as any substance or combination of substances presented as having properties for treating or preventing disease in human beings [7]. Unlike cosmetics a marketing authorization for medicinal products has to be issued by the competent authorities.

The medicine consists of active pharmaceutical ingredients (API) and excipients. An API is defined by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidance as any substance or mixture of substances intended to be used in the manufacture of a drug product and that, when used in the production of a drug, becomes an active ingredient in the drug product. Such substances are intended to exert a pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease or to affect the structure and function of the body [8]. In turn, excipients are any constituent of a medicinal product other than the active substance and the packaging material. The excipients may have several functions, for example in the manufacturing process (e.g., glidants, lubricants and binders), the release of the drug from the dosage form (e.g., disintegrants) or simply to improve handling and dosing uniformity (e.g., fillers or diluents), to provide drug stability (e.g., antioxidants), good taste (e.g., sweetening agents and flavours), or appearance (e.g., colorant and coating components) [9].

Pharmaceutical products are formulated with a fairly limited number of substances in comparison with cosmetics. The raw materials used in the manufacture of a medicine have to meet a series of requirements whether they are active substances or excipients (Figure 1). Pharmacopeias contain monographs that describe both the individual and general quality standards for ingredients, dosage forms and methods of analysis for medicines [10].

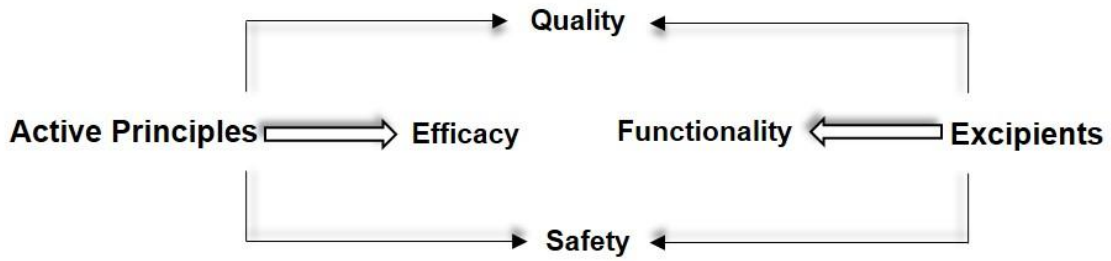

Figure 1: Main requirements for pharmaceutical formulations. Adapted from [11].

With regard to safety assessment, the development of a pharmaceutical is a stepwise process involving an evaluation of both non-clinical and human safety information. Human clinical trials are conducted to demonstrate both the efficacy and safety of a pharmaceutical, starting with a relatively low exposure in a small number of subjects. Clinical trials in which exposure usually increases by dose, duration, and/or size of the exposed patient population are also performed [12]. Regarding non-clinical safety evaluation both animal models as well as *ex vivo* and *in vitro* preparations can be used as test systems [13].

For excipients, if a precedence of use can be shown in applications where there is human exposure, the safety of the material might already be appropriate for potential application as an excipient in the pharmaceutical industry. If there is no precedence of use in a drug product, then the material is to be considered a new excipient and there are a number of conditions set out by the regulatory authorities to allow for its use, including safety assessment [14].

The scientific guidelines available for the non-clinical safety evaluation of raw materials for medicines and cosmetics are presented on Table 1.

Table 1: Scientific guidelines applied to cosmetic and pharmaceutical formulations regarding non-clinical safety assessment

Medicines	Cosmetics
Guideline on non-clinical local tolerance testing of medicinal products (European Medicines Agency, 2014)	The SCCS's notes of guidance for testing of cosmetic ingredients and their safety evaluation 9th revision (European Commission, 2015)
Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals (M3(R2)) (ICH, 2009)	Cosmetics Europe: guidelines for the safety assessment of a cosmetic product (Cosmetics Europe, 2004)
Guideline on repeated dose toxicity (European Medicines Agency, 2010)	Guidance on the safety assessment of nanomaterials in cosmetics (European Commission, 2012)
Testing for carcinogenicity of pharmaceuticals (S1B) (ICH, 1997)	
Photosafety evaluation of pharmaceuticals (S10) (ICH, 2013)	
Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use (S2(R1)) (ICH, 2011)	
Detection of toxicity to reproduction for medicinal products & toxicity to male fertility (S5(R2) (ICH, 2000)	
Non-clinical guideline on drug-induced hepatotoxicity (European Medicines Agency, 2008)	
Note for guidance on safety pharmacology studies for human pharmaceuticals (European Medicines Agency, 2006)	
Guidance for industry: nonclinical studies for the safety evaluation of pharmaceutical excipients (Food and Drug Administration, 2005)	

1.1.1 Toxicological testing of raw materials for topical formulations

Procedures for the skin testing of new chemicals and finished products have been evolving in the face of technological advancements and political pressure. The evaluation of the skin irritation potential is such an example. It was usually performed with the method of John Draize [15]. This method consisted in the application under occlusion of test compounds on rabbit skin and subsequent evaluation of irritant effects [16]. Recently, measures such as prohibition of execution of cosmetics animal testing led to the development and validation of alternative *in vitro* methods using reconstructed human epidermis. Currently, several *in vitro* skin irritation tests are officially validated, namely EpiSkin™, Modified Epiderm™ Skin Irritation Test and SkinEthic™ Reconstructed Human Epidermis [5]. These models use normal human keratinocytes that, during culturing, form a multi-layered epidermis including a stratum corneum at the top, functioning as a barrier [17].

Nanomaterials (NMs) are a particular case of cosmetic ingredients. Nanotechnologies are a booming business and include the development and production of nanosized engineered particles, fibers, coatings, etc., collectively referred to as NMs [18]. According to Regulation (EC) No 1223/2009 nanomaterial means an insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm [2]. Currently, these nanosystems can be found in many cosmetic products such as sunscreens, creams, tooth pastes, shampoos and cleansing agents. Additionally, nanotechnology has been widely studied for medical and pharmaceutical applications [19, 20]. Safety data with special considerations to the properties of a specific nanomaterial is required for its risk assessment. A guidance document on the safety assessment of nanomaterials in cosmetics was published [21].

On Table 2 a set of toxicological requirements and their scope of application (cosmetics or medicines) are listed. Safety requirements for cosmetic ingredients are addressed in the “Notes of Guidance for Testing of Cosmetic Ingredients and their Safety Evaluation” [5]. Basic requirements for cosmetic substances present in finished cosmetic products include acute toxicity (oral, dermal or inhalation), skin and eye irritation, sensitization and mutagenicity data. For inclusion of a substance in one of the annexes to Regulation (EC) No 1223/2009, in general, points 1. to 6. are considered the minimal base set requirements. However, when considerable oral intake is

expected or when the data on dermal/percutaneous absorption indicate a considerable penetration of the substances through the skin, points 7., 8. and 9. may become necessary, as well as specific additional genotoxicity and/or mutagenicity data. Photo-induced toxicity data are specifically required when the substance is present in a cosmetic product that is expected or intended to being used on sunlight-exposed skin [5].

The nonclinical safety study recommendations for the marketing approval of a pharmaceutical usually include repeated dose toxicity studies, reproduction toxicity studies, genotoxicity studies, local tolerance studies, and for drugs that have special cause for concern or are intended for a long duration of use, an assessment of carcinogenic potential. Other nonclinical studies include pharmacologic studies for safety assessment (safety pharmacology) and pharmacokinetic (absorption, distribution, metabolism, and excretion-ADME) studies. The complete evaluation of dermal tolerance for pharmaceutical products intended for administration to the skin requires a repeated dose dermal tolerance test, and evaluation of the sensitizing potential, a photosafety evaluation should also be undertaken [13]. As a general rule, the formulation that is intended to be used clinically should be used in all tests.

Table 2: General toxicological requirements for cosmetic ingredients or active substances of topical medicines

General toxicological requirements	Definition
1.Acute toxicity (if available) **C**	Adverse effects on health which may result from a single exposure to a substance via the oral, dermal or inhalation route.
2.Corrosivity and Irritation (skin and eye) **C**	Corrosion is the potential of a substance to cause irreversible damage while Irritation causes reversible damage.
3.Skin sensitization **C,M**	An agent that is able to cause an allergic response in susceptible individuals.
4.Dermal/percutaneous absorption **C**	Passage of compounds across the skin, including penetration, permeation and resorption.
5.Repeated dose toxicity **C,M**	Occurs as a result of repeated daily dosing with, or exposure to, a substance for a specific part of the expected lifespan of the test species.
6.Mutagenicity/genotoxicity **C,M**	Mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms and genotoxicity is a broader term and refers to processes which alter the structure, information content or segregation of DNA.
7.Carcinogenicity **C,M**	Substances are defined as carcinogenic if they induce tumors or increase their incidence, malignancy or shorten the time of tumor occurrence.
8.Reproductive toxicity **C,M**	Adverse effects induced by a substance on any aspect of mammalian reproduction.
9.Toxicokinetics **C,M**	Describes the time-dependent fate of a substance within the body and includes absorption, distribution, biotransformation and/or excretion.
10.Photo-induced toxicity **C,M**	Occurs as a result of the exposure of the substance to a non-cytotoxic dose of UV/visible light.
11.Safety pharmacology **M**	The core battery of safety pharmacology studies includes the assessment of effects on cardiovascular, central nervous, and respiratory systems, and should generally be conducted before human exposure.

C refers to Cosmetics and **M** to Medicines

Dermal tolerance is an important requirement for topical formulations. Animal models or *in vitro* tests using reconstructed human epidermis have been used for non-clinical dermal tolerance assessment of raw materials for the cosmetic and pharmaceutical industry [13]. Simple cytotoxicity tests can be of value early in the discovery process of new drugs or active substances for the pharmaceutical industry. These assays can be added to the battery of information (pharmacologic efficacy, selectivity, and ADME properties) that is used to differentiate between chemical series or aid in compound selection for safety tolerance assessment of drugs in animal studies [22].

Numerous *in vitro* screening assays have been developed to measure specific biological activities of chemicals in specific organs or cell types with an eye to elucidating mechanisms of action. For the purposes of hazard assessment, biological activity in an *in vitro* system can identify a mechanism of action or response that could be extrapolated to an *in vivo* end point [23].

In the following section an approach to *in vitro* cytotoxicity assays is done.

1.2 *In vitro* cytotoxicity assays

The detection of cytotoxicity is crucial in many biological fields, e.g. in toxicology and in pharmaceutical and cosmetic fields for the assessment of toxic effects elicited by chemicals, drugs or cosmetic ingredients, respectively, before they are release for use by the public.

A variety of assays have been developed and used for the measurement of cell viability or toxicity and the *in vitro* proliferation. It is important to make a distinction between cytotoxicity or cell viability and proliferation. Cell proliferation is the measurement of the number of cells that are dividing in a culture. One way of measuring this parameter is by performing clonogenic assays, a defined number of cells are plated onto the appropriate matrix and the number of colonies that are formed after a period of growth are enumerated or measurement of DNA synthesis as a marker for proliferation (e.g., 3 [^{3}H]-thymidine or 5-bromo-2'-deoxyuridine) [24]. Definitions of cytotoxicity or cell viability vary depending on the nature of the study. Cytotoxicity is the cell-killing property of a chemical (e.g., cosmetic or pharmaceutical) or a mediator cell (e.g., cytotoxic T cell), independent from the mechanisms of death, while cell viability can be taken as the number of live cells [24].

The need for reliable, easy to handle and fast cytotoxicity tests led to the development of several assays which are now routinely used and available to detect

cytotoxic effects in *in vitro* cellular systems [25]. These assays have been devised to examine and measure cessation of a broad variety of parameters associated with biochemical events necessary for sustaining viability and/or evidence of changes in membrane integrity leading to cellular disintegration [26].

To choose an appropriate *in vitro* cytotoxicity assay, different parameters as: the cell type, culture conditions applied, test compounds, detection mechanism, specificity, and sensitivity have to be considered. Cell-toxicity may be assessed by morphological changes or by alterations in membrane permeability and/or physiological state inferred from the exclusion of certain dyes or the uptake and retention of others [26, 27].

In the following topic, a brief description, as well as, the advantages and disadvantages of the most used cytotoxicity assays will be described.

1.2.1 Evaluation of cell membrane permeability

Widely used standard methods for measuring cell membrane permeability are based on the uptake or exclusion of molecules with particular light-absorbing or fluorescent properties. An example is the uptake of dyes such as Trypan Blue, Neutral Red and Propidium iodide, or the release of cytoplasmic enzyme or of artificial label (Figure 2). This evaluation is based on the fact that viable cells have an intact plasma membrane while dead cells have a damaged plasma membrane [24].

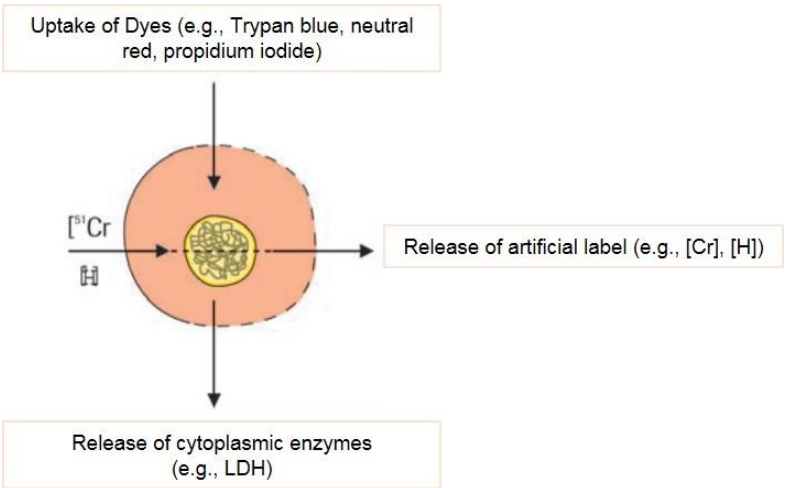

Figure 2: Schematic illustration of the three basic principles to assess cell membrane integrity. Adapted from [24].

The advantages of such permeability assays are that they are performed easily and with use of multi-wells plates, many dilutions and/or many compounds can be tested rapidly [28]. On the other hand, a serious disadvantage of these assays is that the initial sites of damage of many cytotoxic agents are intracellular. Consequently, cells may be irreversibly damaged and committed to die and the plasma membrane is still intact. Thus, these assays tend to underestimate cellular damage when compared to other methods. Despite this fact, some permeability assays have been widely accepted for the measurement of cytotoxicity [24].

1.2.1.1 Calcein-Acetoxymethyl assay

The Calcein-acetoxymethyl (AM) assay was developed by Lichtenfels *et al.* [29]. Calcein-AM is a dye that passively crosses the cell membrane and is frequently used to stain viable cells [30]. Inside the cells it is converted by intracellular esterases into a polar lipid-insoluble fluorescent product, calcein, that is retained by cells with intact membranes but that is released by damaged ones, producing an intense green signal (Figure 3).

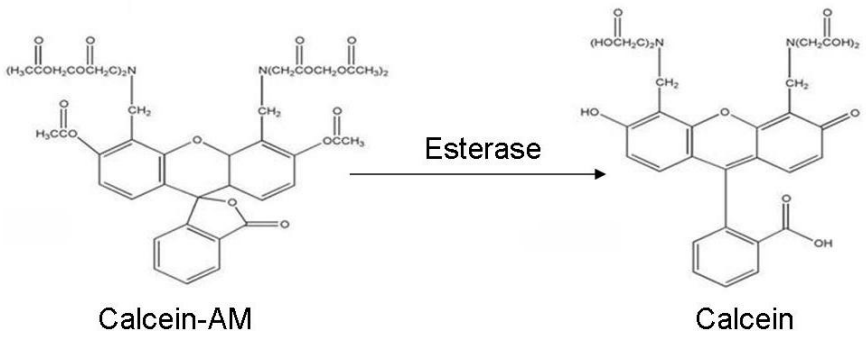

Figure 3: Mechanism of conversion of Calcein-AM to Calcein by esterases. Adapted from [31].

Release of calcein by damaged cells in the supernatants can be measured rapidly and with a high level of sensitivity by a fluorimeter or flow cytometry [32].

1.2.1.2 Fluorescein Diacetate uptake assay

Another assay for evaluation of cell membrane permeability involves the uptake of the fluorescein diacetate (FDA), one dye permeable to the cell membrane. The assay is based on the hydrolysis of the dye, FDA, by esterases in cells with intact plasma membranes [33].

A non-polar substrate and non-fluorescent, FDA, is incorporated into live cells and is converted into polar and green fluorescent product, fluorescein, by cellular hydrolysis (Figure 4). Under the appropriate excitation and emission wavelengths, viable cells fluoresce as green and this feature can be measured either by visual counting with a fluorescence microscope or by flow cytometry [34].

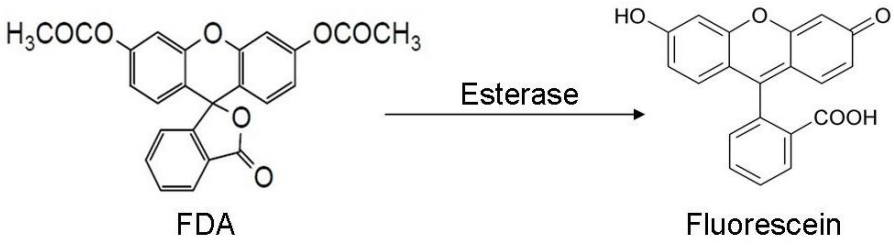

Figure 4: Mechanism of conversion of FDA to Fluorecein by esterases. Adapted from [35].

One advantage of using FDA is the speed at which results can be obtained, as the previous method. The disadvantages that can arise when using this test are the false negatives, when FDA is converted to fluorescein in the absence of live cells or otherwise when the fluorescein that is produced by live cells may not be detectable if its fluorescence is quenched [36].

1.2.1.3 Lactate Dehydrogenase leakage assay

Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme which serves as an indicator of lytic cell death since it is readily released into extracellular medium following cellular membrane damage resulting from apoptosis or necrosis [37]. There are other cellular enzymes, such as adenylate kinase and glucose-6-phosphate dehydrogenase that are used as cell death markers. However, these are not stable and only LDH does not lose its activity during cell death assays. Therefore, cytotoxicity

assays based on LDH activity are more reliable than other enzyme-based cell death assays [24].

The LDH leakage assay is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD, by the following reaction:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\leftrightarrow} \text{NAD}^+ + \text{lactate}$$

The formation of NADH from the above reaction leads to a change in absorbance at 340 nm. LDH is rapidly released into the cell culture supernatant upon damage of the cell membrane, an indicator of irreversible cell death [38, 39].

Reliability, speed and simple evaluation are some of characteristics of this assay [40]. Although widely accepted as a marker of cell death, it should be noted that this test is simply an index of cell membrane integrity, and in certain circumstances can be positive even when the cell count is not significantly modified [37].

1.2.1.4 Neutral Red uptake assay

The neutral red (3-amino-m-dimethylamino-2-methlphenazine hydrochloride) is a weak cationic dye that has been used previously for the identification of the viable cells in cultures [41].

The neutral red (NR) assay is based on the initial protocol described by Borenfreund and Puerner [42]. This assay focuses on the uptake and subsequent lysosomal accumulation of the supravital dye NR. Cellular uptake of the dye is accomplished by passive transport across the plasma membrane. Accumulation of NR within lysosomes occurs either from the binding of NR to fixed acidic charges, such as those of polysaccharides, within the lysosomal matrix, or from the trapping of the protonated form of NR within the acid milieu of the lysosomes [43].

In damaged or dead cells NR is no longer retained in the cytoplasmic vacuoles and the plasma membrane does not act as a barrier to retain the NR within the cells. It quantifies the number of viable, uninjured cells after their exposure with test agents [39, 44].

This assay does not measure the total number of cells, but it does show a reduction in the absorbance related to loss of viable cells [43].

1.2.1.5 Propidium Iodide exclusion assay

Propidium iodide (PI) is a fluorescent molecule that can only penetrate cell membrane of dying or dead cells and thus identifies dead cells within a population. Upon entering dead cells, PI binds to double-stranded DNA by intercalating between base pairs. As this intercalation is mediated by non-covalent forces, these dyes must remain present in the buffer used to resuspend cells for data acquisition so that dead cells will remain labeled [45]. The uptake of PI by the cells results in orange fluorescence that can be assessed by fluorescence and flow cytometry [46].

1.2.1.6 GF-ACF assay

This assay utilizes the cell-permeant protease substrate glycylphenylalanyl-aminofluorocoumerin (GF-AFC) that penetrates live cells where cytoplasmic aminopeptidase activity removes the gly and phe aminoacids to release aminofluorocoumarin (AFC) and generate a fluorescent signal proportional to the number of viable cells (Figure 5) [47, 48].

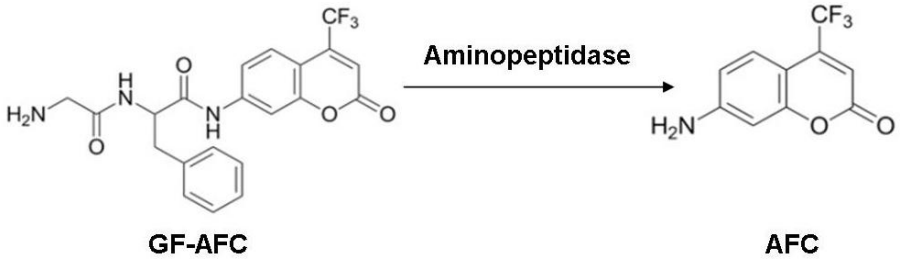

Figure 5: Mechanism of conversion of GF-AFC to AFC by cytoplasmic aminopeptidase activity. Adapted from [47].

The selective detection of viable cells by this method is possible because the proteolytic activity towards the GF-AFC substrate is dependent upon the continued maintenance of membrane integrity. Viability can be measured using a fluorometer. The proteolytic activity decays within seconds after a cytotoxic event, so non-viable cells do not contribute appreciably to fluorescence generation [48].

One of the advantages of the GF-AFC substrate is that it is relatively non-toxic to cells in culture.

1.2.1.7 Trypan Blue exclusion assay

One of the earliest methods for assessing cytotoxicity was trypan blue exclusion assay, which is still widely used today.

The trypan blue is a vital stain used to stain dead cells or tissues. When membrane integrity of the cells is compromised, there is an uptake of the dye into the cells so that viable cells, which are unstained, appear clear with a refractile ring around them and nonviable cells appear dark blue colored [25, 28]. This traditional method involves manual staining and use of a hemocytometer for counting. Recent advances in instrumentation have led to a number of semi- or fully automated systems that can increase the throughput and accuracy of this technique [49].

This method is simple, quick, inexpensive, and requires only a small fraction of total cells from a cell population. However, trypan blue staining cannot be used to distinguish between the healthy cells and the cells that are alive but with impaired cell functions.

1.2.2 Evaluation of metabolic activity

One parameter used as the basis for cytotoxicity assays is the evaluation of metabolic activity of the viable cells including assays based on tetrazolium and resazurin reduction [26]. The metabolic assay do not provide direct information about total cell numbers, but measure the viability of a cell population relative to control. One potential disadvantage of these metabolic assays is that there is no differentiation between cells that are actively dividing and those that are quiescent which may result in an over-estimation of cell number [27].

1.2.2.1 AlamarBlue® reduction assay

AlamarBlue® is an important and sensitive oxidation-reduction indicator that is used to evaluate metabolic function and cellular health. The resazurin (oxidized form) is a blue weakly fluorescent indicator dye that changes into highly fluorescent pink resorufin (reduction form) in response to irreversible chemical reduction (Figure 6) [50].

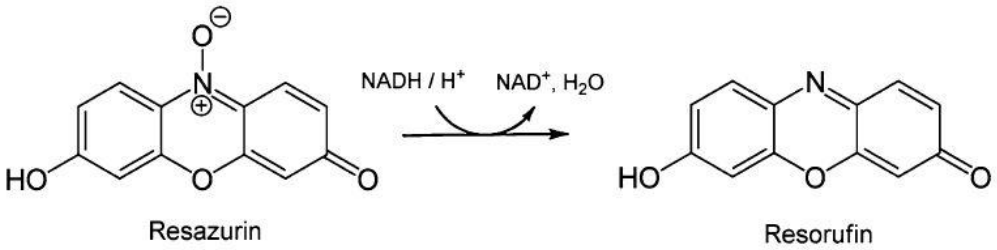

Figure 6: Mechanism of reduction of resazurin to resorufin [50].

Inside the cell, AlamarBlue® undergoes enzymatic reduction in mitochondria due to the activity of enzymes such as flavin mononucleotide dehydrogenase, flavin adenine dinucleotide dehydrogenase, nicotinamide adenine dehydrogenase, and cytochrome [51]. It was also noted that cytosolic and microsomal enzymes have abilities to reduce resazurin [52]. The pink resorufin is secreted outside the cells to the medium which results in visible colour change from blue to pink. The rate of reduction based on colour change can be quantified colorimetrically or fluorometrically and reflects the number of viable cells [50].

AlamarBlue® is considered as very sensitive technique, non-toxic to cell cultures and is less likely to affect normal metabolism by not interfering with the electron chain [50, 53].

1.2.2.2 Tetrazolium Salts reduction assay

A variety of tetrazolium compounds have been used to detect viable cells. The most frequently used tetrazolium salt is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [27].

The MTT reduction assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS) [54]. This assay is used to determine the level of metabolic activity in eukaryotic cells. It is based on the conversion of soluble tetrazolium into insoluble formazan crystals reduction (Figure 7) by cellular mitochondrial and cytosolic enzymes of actively growing cells [25, 26]. The formazan product is impermeable to the cell membranes and requires solubilization of product.

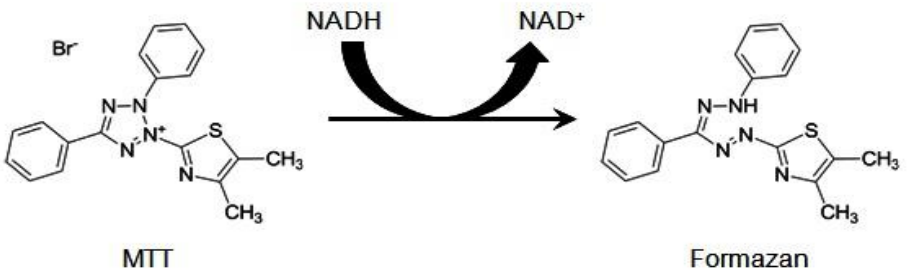

Figure 7: Structures of MTT and colored formazan product [47].

Problems surrounding MTT insoluble formazan product prompted investigation and the creation of alternative tetrazoliums with different properties, namely to generate formazan products that are directly soluble in cell culture medium [26]. Tetrazolium compounds which fit this category include 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), sodium 3′-[1-phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitrobenzene) sulfonic acid hydrate (XTT) and 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) [55, 56]. These derivatives are reportedly more sensitive and have fewer steps as the converted product is released into the medium, meaning no step is required to dissolve the insoluble product (a requirement when using MTT). However, MTT is very robust and is metabolized by most cell types, whereas some of the newer alternatives are not suitable for all cells [24, 25].

Thus, selection of the most appropriate tetrazolium salt as an indicator of metabolic activity is important.

1.2.3 Evaluation of adenosine triphosphate content

The nucleotide adenosine triphosphate (ATP) that is present in all metabolically active cells plays an important role in energy exchange in biological systems. It functions as the principal immediate donor of energy. ATP has been used as a tool for the functional integrity of living cells, since all cells require ATP to remain alive and carry out their specialized function [57, 58]. During the process of cell death, there is a loss of ability to synthesize ATP and endogenous cytoplasmic ATPases rapidly remove any remaining ATP [26].

Measuring the amount of ATP from samples of cells in culture has been widely accepted as a valid marker of the number of viable cells present under most experimental conditions [26]. Many methods have been used for evaluation of ATP content, but the most successful technique is the bioluminescent assay, because of its high sensitivity and wide dynamic range [57]. The disadvantage of this method is the luminescence-readout, which could be influenced by quenching side effects in the samples [59].

1.2.3.1 Bioluminescent assay

In the bioluminescent assay, the enzyme, luciferase catalyzes the formation of light from ATP and luciferin [44], by the following reaction:

$$ATP + D\text{-}Luciferin + O_2 \rightarrow Oxyluciferin + AMP + PPi + CO_2 + Light \quad (560nm)$$

Cellular ATP can be measured by direct lysis of the cells with a suitable detergent, the released ATP is then free to react with the luciferin-luciferase, leading to light emission. The emitted light intensity is linearly related to the cell ATP concentration and can be measured using a luminometer [58].

The principal advantages of ATP detection were shown to be its sensitivity and reproducibility [60]. The disadvantages of this assay include that it kills the cells and so the sample generally cannot be used for other purposes after treatment with the reagent.

1.2.4 Evaluation of protein content

The evaluation of protein content is an indirect measurement of cell viability. The most used assays are: bicinchoninic acid and sulphorhodamine assays.

1.2.4.1 Bicinchoninic Acid assay

The bicinchoninic acid (BCA) assay also know Smith assay was described by Smith [61]. The principle this assay is similar to the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+} [62].

In this assay, the amount of reduction is proportional to the protein present, exhibited by a color change of the sample solution from green to purple in proportion to protein concentration, which can then be measured using colorimetric techniques or absorbance. The major advantage of the BCA is that it is stable under alkali conditions [63]. One disadvantage of the BCA assay is that it is susceptible to interference by some chemicals present in protein samples, including reducing agents or copper chelators [64].

1.2.4.2 Sulphorhodamine assay

The Sulphorhodamine (SRB) assay is a popular *in vitro* cytotoxicity assay developed by Skehan *et. al*. [65]. This assay is based on binding of the SRB to basic aminoacids of cellular proteins and colorimetric evaluation provides an estimate of total protein mass, which is related to cell number [66].

The advantages of this test include better linearity, higher sensitivity, a stable end point that does not require time-sensitive measurement and lower cost [67].

Cytotoxicity assays described herein are just a few of the commonly used assays. These can be used as a prediction of the adverse effects of test compounds on living systems, for the detection of toxic thresholds, and expansion of experimental data sets to include multiple toxicity end-point analysis, which are required for any robust screening regime [27].

In Table 3, the cytotoxicity assays described in this topic are presented, grouped by the parameter evaluated, as well as the principle of detection of each test.

Table 3: Summary of the most used in vitro cytotoxicity assays

Evaluated parameter	Cytotoxicity assay	Detection principle
Cell membrane permeability	Calcein-AM	Florescence or Flow cytometry
	FDA uptake	Fluorescence or Flow cytometry
	LDH release	Absorbance
	NR uptake	Absorbance
	PI exclusion	Fluorescence or Flow cytometry
	GF-AFC	Fluorescence
	Trypan Blue exclusion	Hemocytometer
Metabolic activity	AlamarBlue® reduction	Absorbance or Fluorescence
	Tetrazolium salts reduction	Absorbance
ATP content	Bioluminescent	Luminescence
Protein content	BCA	Absorbance
	SBR	Absorbance

1.3 Use of cytotoxicity assays in safety assessment of raw material for cosmetic and topical pharmaceutical formulations

The skin is the first site of contact for many compounds, including ingredients of cosmetics or dermal pharmaceuticals. Skin, the major human organ, is a heterogeneous membrane: lipophilic on its surface and hydrophilic in its deeper layers. It forms a remarkable protective barrier against the external environment, helping to regulate temperature and fluid balance, keeping out harmful microbes or chemicals and offering some protection against sunlight and oxidation [68, 69].

Histologically, the skin has three different layers – epidermis, dermis and hypodermis (Figure 8) – that have distinct composition and functions.

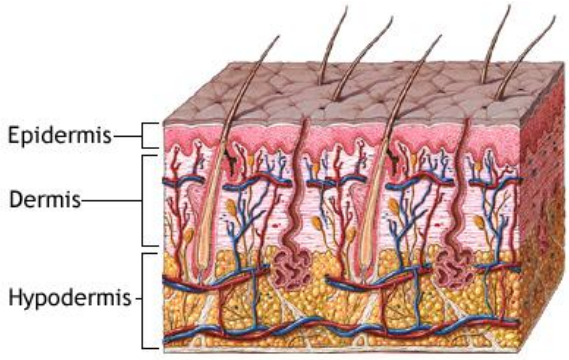

Figure 8: Structure of skin tissue [70].

- Epidermis

Epidermis is the outermost structure of human skin and is relatively thin depending on body part. The palmar and plantar areas of the feet present the thickest epidermis. This tissue is a stratified squamous epithelial film and is an avascular layer, being fed through the diffusion of nutrients from the dermis. It consists of several layers (listed from outermost to innermost layer) namely the stratum corneum, stratum granulosum, stratum spinosum, and stratum basalis (Figure 9). In the thickest areas of the skin, another layer called stratum lucidum is visible, being located immediately below the stratum corneum [71-74].

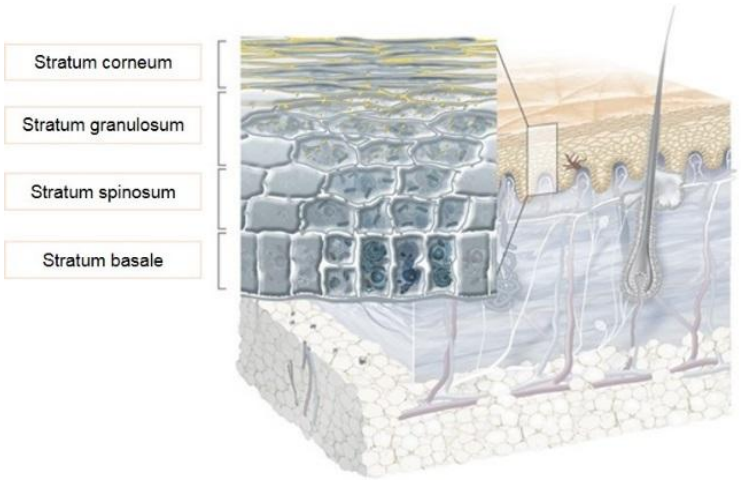

Figure 9: Structure of the epidermis. Adapted from [75].

Keratinocytes are the predominant cells in the epidermis and multiply continuously, safeguarding the constant renewal of the epidermis. On the process of migration of keratinocytes from the stratum basale to the stratum corneum, named keratinization, the cells are undergoing changes in form and chemical composition, and become rich in keratin. Keratinocyte transit time is about 14 days and turn over time within stratum corneum is also around 14 days, although certain inflammatory conditions can affect these turn over times [71]. This way, the cells eventually die forming an outer layer, which imparts strength and forms an impervious barrier on the skin. The mature keratinocytes are formed by a high amount of soluble, low molecular weight substances which effectively bind water to prevent dehydration of the skin. In addition to the keratinocytes, the epidermis has other cell types: melanocytes, Langerhans cells, and Merkel cells. Melanocytes are branched cells, located between the keratinocytes of the basal layer and are responsible for production of melanin, which is important for the protection of skin against ultra-violet radiation and free-radicals. Langerhans cells are found throughout the epidermis and show defense immune functions. Merkel cells are especially found on thick skin areas and at the fingertips, and are responsible for cutaneous sensation [73, 76].

- Dermis

The dermis is a connective tissue layer and contains a collagen- and elastin-containing extracellular matrix, primarily composed of type I collagen, as well as an abundance of blood vessels and specialized nerve endings, thus providing structural and nutritional support to the skin [68, 72, 77]. The dermis contains four major resident cell types: fibroblasts, dermal dendritic cells, macrophages, and mast cells. Various infiltrative inflammatory cells can also be found in the dermis under different conditions [72].

The dermis has two distinct layers: the reticular dermis and papillary dermis. The reticular dermis is the main layer of the dermis and is continuous with the hypodermis and has a predominance of elastic and collagen fibers. The papillary dermis is composed of papillae extending towards the epidermis. Compared with the first, the papillary dermis has fewer fibers and more cells. This layer of skin also contains numerous blood vessels, to provide nutrients to the epidermis, removing waste products and helps to regulate the body temperature [73].

- Hypodermis

The hypodermis or subcutaneous fat layer is situated beneath the dermis and connects this with the underlying organs. It is primarily composed of well-vascularized adipose tissue and loose connective tissue, which insulates the body and provides both thermoregulatory and mechanical functions [71-73, 78].

The assessment of cytotoxicity in skin cells can be taken as a measure of the skin irritation potential. The evaluation of the skin irritation potential is essential to ensuring the safety of human in contact with a wide variety of cosmetic and/or pharmaceutical substances.

Traditionally, the assessment of skin irritation involved the use of laboratory animals [79, 80]. However, since the Regulation (EC) 1223/2009 has come into force, animal testing of cosmetic and ingredients was banned and the study of their safety has moved towards *in vitro* methodologies. Cultured human skin cells are a potentially useful model for skin irritation testing. *In vitro* cultures of the monolayer using primary

human keratinocytes, dermal fibroblasts or immortalized epidermal cell lines have been used in various *in vitro* irritation assays [81].

In vitro cytotoxicity assays have the advantage of being relatively inexpensive, standardized, sensitive and allow for rapid screening of compounds [82].

The most common *in vitro* cytotoxicity assay is MTT [83]. This assay is used in primary human keratinocytes [84, 85], immortalized keratinocytes [86], melanocytes [87] and fibroblasts [88]. Reconstructed human epidermal skin models are being used increasingly for safety testing of chemicals. These models have the advantage of allowing topical application of the compounds, evaluation of formulations and poorly soluble compounds, as well as measurement of local toxicological effects in target cells [89]. MTT reduction assay also is very used in reconstructed human epidermis [90-93].

1.3.1 Analyzed antioxidants raw materials

The skin has an antioxidant defense system that prevents UV-induced oxidative stress. Nevertheless chronic and excessive exposure to UV-radiation leads to oxidative stress and oxidative damage [94]. The topical application of antioxidants has achieved great expression, owing to their ability to prevent or minimize the UV induced-deleterious effects of reactive species on the skin [95].

In this work were analyzed several compounds: resveratrol and its derivatives; resveratrol-loaded lipid and polymeric particles, xanthone and plant extract.

- Resveratrol and its derivatives

The Resveratrol - RSV (3, 5, 4’ -trihydroidroxystilbene) a natural antioxidant compound is a non flavonoid polyphenol, present in different plants (peanuts, pistachio nuts, red grape seeds and skins, red wine, and other plant-derived food products) [96]. Several beneficial effects have been attributed to RSV and its use has been proposed in the treatment or prevention of different diseases [97].

The resveratrol glicoside – RSV-Gli (3, 5, 4’ -trihydroidroxystilbene-3-β-D-glucopyranoside) is an natural precursor of RSV and known for its favorable therapeutic properties: cardiovascular effects, neuroprotection, anti-inflammatory and immunoregulatory effects, anti-oxidation, anti-tumor, liver and lung protection [98].

The resveratrol glicoside sulfate - RGS (3, 5, 4' -trihydroidroxystilbene-3-β-D-glucopyranoside persulfate) was obtained by chemical synthesis and demonstrate *in vitro* anticoagulant and antiplatelet activities [99, 100]. In docking study, RGS binds more stably to sirtuin (lower binding free energy) than the RSV. RGS establishes polar interactions with 5 sirtuin residues and stacking interactions with the substrate peptide, whereas RSV establishes only one polar interaction (unpublished results). These results support the interest of this derivative for skin care formulations.

The chemical structure of RSV and its derivatives (RSV-Gli and RGS) is showed in Figure 10.

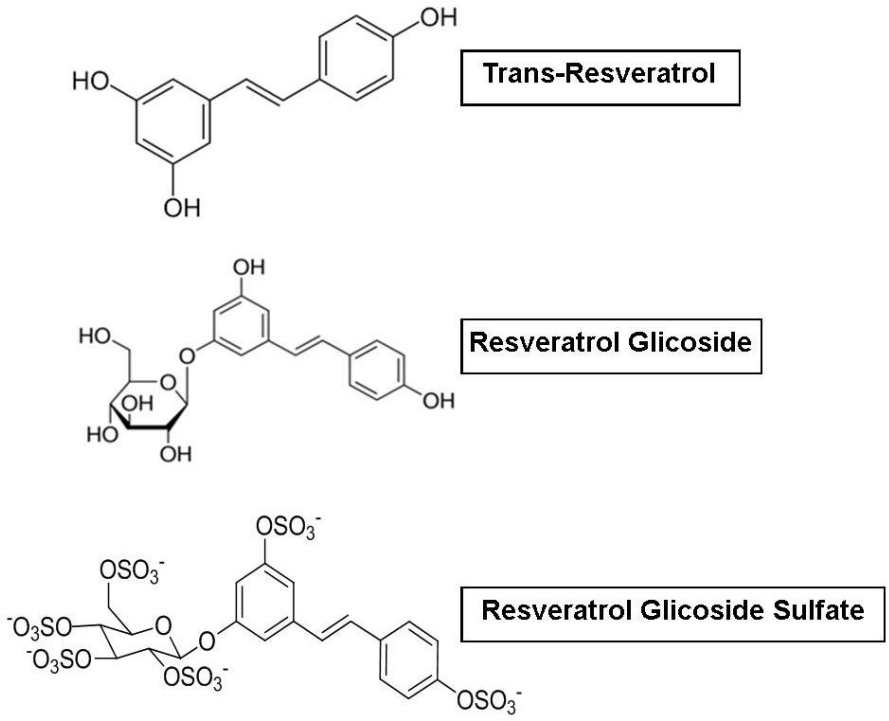

Figure 10: Chemical structure of RSV and its derivatives studied in this work.

- Lipid and polymeric particles

Due to its low chemical stability, poor solubility, inefficient systemic delivery, and low bioavailability [101], RSV was encapsulated in lipid and polymeric particles.

Lipid nanoparticles can be solid lipid nanoparticle (SLN) or nanostructured lipid carriers (NLC) (Figure 11). The first ones, were invented 20 years ago and are prepared from a lipid matrix that is solid at body and room temperature and stabilized by suitable surfactants [102] and NLC are 2nd generation of solid lipid-based colloidal carriers and are composed of a mixture of a solid and a liquid lipid which lipid matrix is solid at room and body temperature [103].

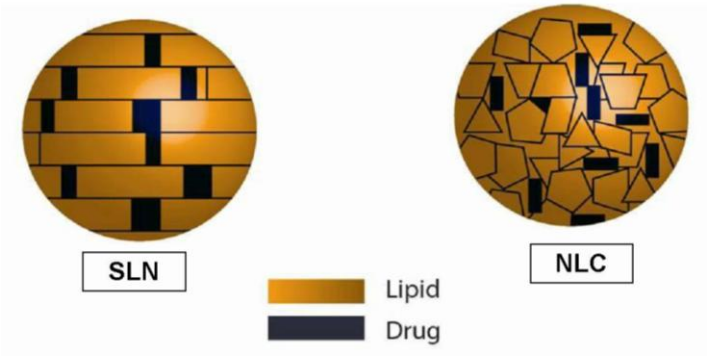

Figure 11: Lipid nanoparticles [104].

The benefits of the lipid nanoparticles include the employment of biocompatible and biodegradable materials, which lead to a low or total absence of toxicity and the ability to transport lipophilic drugs [104].

On the other hand, polymeric particles are solid colloidal particles that are dispersed in the liquid and upon water evaporation, they get well organized on the dosage form solid surface to form close-packed arrays. Eudragit® RS 30D was the utilized polymer. It is the synthetic homopolymer of poly(acrylate) and poly(methacrylate) [105].

- Chlorogenic acid persulfate and Ascorbic acid persulfate

Chlorogenic acid persulfate (ACS) and ascorbic acid persulfate (ASS) (Figure 12) are compounds obtained by chemical synthesis with *in vitro* anticoagulant, antiplatelet and antioxidant activities [99, 100].

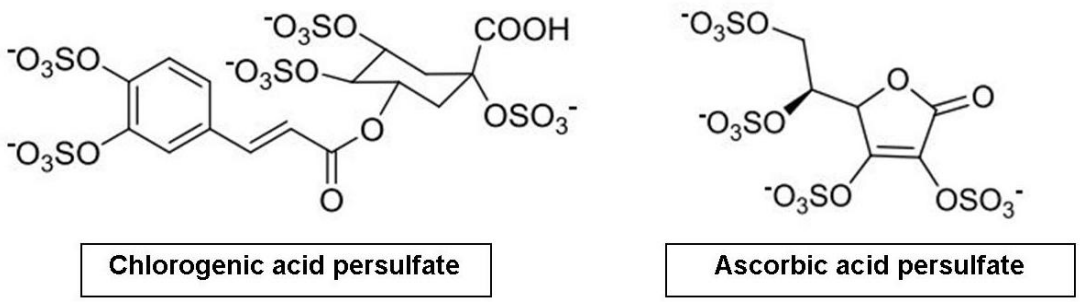

Figure 12: Chemical structure of ACS and ASS studied in this work.

- Xanthone

Chemically, xanthones are a class of oxygenated heterocyclic compounds with a dibenzo-γ-pyrone scaffold [106]. Xanthones can be considered as "privileged structures", considering that their scaffold contains a rigid ring system allowing the insertion of a variety of substituent's capable of interacting with several biological targets. Many xanthones with phenolic groups have been described for their antioxidant properties [107]. These properties have been implicated also with their anti-inflammatory and cancer chemopreventive activities [108]. In the present work, a 1,2-dihydroxyxanthone (Figure 13) was used.

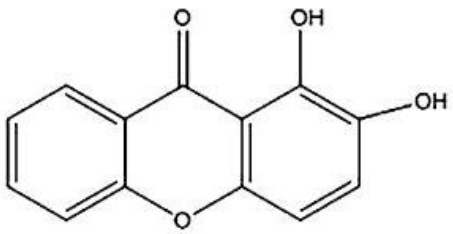

Figure 13: Chemical structure of 1,2-dihydroxyxanthone studied in this work.

- *Castanea sativa* leaf extract

Many natural compounds have a wide range of biological activities including antioxidant, chemopreventive, anti-inflammatory, neuroprotective, and cardioprotective effects [109].

Castanea sativa (C. sativa) is a species of the flowering plant family *Fagaceae*, mainly cultivated in temperate regions like Asia, North of Africa and Southern Europe. Its leaves are used in folk medicine to treat cough, diarrhea and rheumatic conditions, lower back pain, and stiff joints or muscles [110].

As in other medicinal plants, some of the active substances have been identified as phenolic compounds. The scavenging ability has been reported in several studies for different ROS, such as superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), which are implicated in skin aging [111]. The utilization of hydroalcoholic extracts from *C. sativa* leaves (which contain rutin, ellagic acid, hyperoside, isoquercitrin and chlorogenic acid) in cosmetic compositions has been considered safe and suitable for preventing and treating photoaging [112].

2. AIM

The aim of this Master's dissertation was to study the cytotoxicity of a series of ingredients with potential interest for topical application and determine the concentration range for each compound that can be used in future assays to confirm the potentialities of these raw materials for application on the skin. For this purpose we used ingredients of natural origin, ingredients obtained by chemical synthesis and also the ingredient loaded in lipid nanoparticles and polymeric microparticles and five distinct cytotoxicity assays were employed.

3. MATERIALS AND METHODS

3.1 Materials

Cetyl palmitate pellets and Eudragit® RS 30D (Poly(ethyl acrylate-co-methyl methacrylate-co-trimethylammonioethyl methacrylate chloride)) were purchased from Gattefossée SA (France) and from Evonik (Germany), respectively. Resveratrol (RSV) was purchased from Fragon (Brazil). Ethanol (EtOH) and Methanol were supplied by Aga (Portugal) and Merck KgaA (Germany), respectively. Polysorbate 60 (Tween® 60), Miglyol® 812 (caprylic/capric triglyceride) and glycerin were purchased from Acofarma (Spain). Formic acid and Acetic acid glacial were purchased from Panreac química SA (Spain). Immortalized human keratinocyte (HaCaT) cell line was obtained from Cell Lines Service (CLS) (Germany). Dulbecco's Modified Eagles's Medium (DMEM) with 4,5 g/L D-glucose and pyruvate, Inactivated Fetal Bovine serum (FBS), Penicillin-Streptomycin solution, Dulbecco's Phosphate Buffered Saline (DPBS) without calcium chloride and magnesium chloride, 0,25% trypsin-EthyleneDiamineTetraacetic Acid (EDTA) solution and Triton™ X-100 were supplied by Gibco® by Life Technologies (USA). Dimethyl sulfoxide (DMSO), Trypan Blue 0,4% solution and Neutral Red (NR) solution were purchased from Sigma-Aldrich® (USA). AlamarBlue® was provided by Thermo Scientific™ (USA). MTT and Propidium iodide were bought to Life Technologies (USA). The water used in all experiments was purified water obtained using a Direct-Q® Water Purification System (Merck Millipore, Darmstadt, Germany) with a reverse osmosis process.

3.2 Methods

3.2.1 Preparation of the *C. sativa* leaf extract

For the preparation of the *C. sativa* leaf extract a method optimized by Almeida [113] was utilized. *C. sativa* leaves were collected during July 2003 in Mirandela and dried at room temperature for 3 weeks, to obtain a moisture content of less than 10%. Dried leaves (8 g) were grounded (500 µm) and extracted five times (5 x 200 mL) with ethanol (EtOH):water (7:3) at 40°C under magnetic stirring (10 min, 500 rpm). Each

alcoholic extract was filtered using a glass filter G4 funnel (5–15 µm porosity, Schott, Germany), and gathered in a kitasato flask. Ethanol was evaporated at 40°C under vacuum with a rotary evaporator (R-114, Büchi, Switzerland). Finally, the resulting aqueous mixtures were lyophilized (SP Scientific, Virtis wizard 2.0, USA) for 24 hours, generating the final dry extract, which was kept in an amber flask at room temperature in a desiccator. The basic steps of this preparation method are described in Figure 14.

Figure 14: Overview of basic steps of *C. sativa* leaf extract preparation.

3.2.1.1 Qualitative analysis of the phenolic composition of *C. sativa* leaf extract

In order to confirm the presence of phenolic compounds of interest, the *C. sativa* EtOH:water (7:3) leaf extract was analysed using High Performance Liquid Chromatography (HPLC) (Dionex Corporation, Sunnyvale, USA) by comparing retention times of compounds present in the extract with retention times of standards previously analysed with the same chromatographic conditions.

The lyophilized extract was dissolved in EtOH:water (7:3) and filtered (0.22 µm) before being analysed. The sample was injected on a C18 Waters Spherisorb® ODS2 0.46x25x5µm column equipped with a Waters Spherisorb® pre-column (Waters Corporation, EUA) at 20°C, with detection at 280 nm, 320 nm and 350 nm. Organic phase was methanol and aqueous phase was water/formic acid (19:1 (v/v)). The separation was obtained at a flow rate of 0.9 mL/min with a gradient program described on Table 4.

Table 4: Gradient program used for identification of phenolic compounds of *C. sativa* EtOH:water (7:3) leaf extract

Time (min)	Aqueous phase	Organic phase
0	95	5
3	85	15
13	75	25
25	70	30
39	55	45
42	55	45
44	50	50
47	45	55
50	30	70
56	25	75
60	20	80

3.2.2 Preparation of nano and microparticles

For the preparation of lipid nanoparticles (SLN and NLC) a high shear homogenization technique was used based on previous studies performed in our lab [104, 114]. The composition of the SLN and NLC produced in this work are described on Table 5.

The lipid phase with the surfactant and the aqueous phase were heated to 70ºC (lipid's melting point) separately. Then, RSV was dissolved in the lipid phase and the aqueous phase was added to lipid phase. The emulsion was submitted to a homogenization process (Ultra-Turrax T25, IKA®-Labortechnik, Germany) at a stirring speed of 13500 rpm for 2 minutes followed by sonication with a Ultrasonic Processor (VCX 130, Sonics Vibracell, USA) with intensity of 80% for 2 minutes. Finally, the nanoparticles underwent a rapid cooling process with running tap water. Empty SLN and empty NLC were prepared in a similar way, without adding RSV.

The lipid nanoparticles were prepared extemporaneously for all cytotoxicity assays.

Table 5: The formulation parameters of empty and RES-loaded SLN/NLC

	SLN empty	SLN-RSV	NLC empty	NLC-RSV
Cetyl Palmitate (solid lipid at room and physiological temperature)	10%	9.9%	7%	6.9%
Tween 60 (surfactant)	2%	2%	2%	2%
Mygliol 812 (liquid lipid at room and physiological temperature)	-	-	3%	3%
RSV (to be encapsuled)	-	0.1%	-	0.1%
Milli-Q Water	q.s. 100%	q.s. 100%	q.s. 100%	q.s 100%

For the preparation of polymeric microparticles a piezodriven spray generation apparatus (Nano Spray Dryer B-90, Büchi, Switzerland) (Figure 15) was used. Polymeric microparticles containing RSV (Poly-RSV) and empty particles (Poly) were produced. RSV was dissolved in EtOH:water solution (20:80) until saturation and mixed with Eudragit® RS 30D (0.5%). The sample was atomized by piezoelectrically driven membrane vibrations in the small spray cap and millions of precisely sized droplets were ejected each second by the vibrating actuator. These droplets passed through the chamber to form dried solid particles that were electrostatically charged and collected at the collecting electrode. The spray drying experiments were executed using the following settings: spray cap (hole size): 7 µm; inlet temperature: 120 ºC, compressed air flow: 100 mL/min; pressure: 33-35 mbar and spray: 80%.

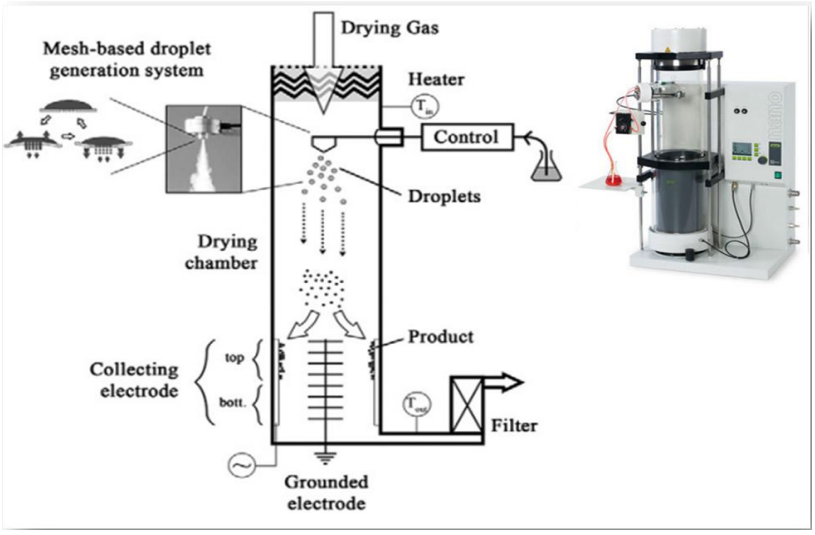

Figure 15: Operating diagram of Nano Spray Dryer B-90. Adapted from [115].

3.2.2.1 Particle size measurements

Particle size of nano and microparticles was analyzed by a laser diffractometry technique (Mastersizer 3000, Malvern, UK). With this technique the intensity of scattered light is measured as a laser beam passes through a dispersed particulate sample. The range of this apparatus is from 0.01 to 3500 µm.

The determination was performed at room temperature using water as a dispersant with a refractive index of 1.33. The refractive index and absorption index were 1.1 and 0.01 for lipid nanoparticles and 1.48 and 0.1 for polymeric microparticles. The sample was added to the dispersant until 5-10% of obscuration (preferably 6%) is reached and tested five times at 1000 rpm. The Dv10, Dv50, Dv90 and Span parameters were obtained by calculating the average of five runs. Only the measurements with residuals and weighted residuals near and below 2% were considered.

3.2.3 Cell Culture

The *in vitro* cytotoxicity was studied using HaCaT cells. The HaCaT adherent and monolayer cell line is an immortal non-cancerous human keratinocyte cell line, which was devised from skin of a Caucasian male with 62 years old.

HaCaT cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 (Heraeus HeraCell 150 Air-Jacketed CO_2 Incubator) in DMEM with 10% FBS and 1% antibiotics (10 000 units/mL penicillin and 10 000 µg/mL streptomycin).

Using an inverted microscope (Motic®, AE2000 Inverted Microscope), cell confluence was assessed. When cells reached 70-80% confluence, subculture was done to prevent cell death. Initially, the culture medium was aspirated and the cells were washed twice with DPBS, then the cells were trypsinized by adding trypsin-EDTA and incubated for 5 to 8 minutes at 37°C in a 5% CO_2 atmosphere. After cell detaching, medium was added in order to block the action of trypsin and cell suspension was centrifuged at 416g for 5 minutes. The supernatant was discarded, the pellet ressuspended in culture medium and cells were counted. For cell counting: 10 µL of cell suspension was added to 90 µL of 0.4% trypan blue vital dye (1:10), ressuspended and placed in a Neubauer chamber (Marienfeld Superior, Germany). Using inverted microscope viable cells (cells that do not incorporate the dye trypan blue) were then counted in a Neubauer chamber. The number of cells in the total volume was calculated with the following equation:

$$\frac{n}{4} \times 10^4 \times \frac{1}{d^{-1}} = \text{number of cells/mL} \quad \text{(equation 1)}$$

where, n is the number of viable cells counted and d is the dilution coefficient.

For cell freezing, DMSO (5% v/v) was used a cryo-preservative to prevent the formation of crystals during the storage phase.

3.2.3.1 Characterization of the HaCaT cell line

One of the key parameters to characterize a cell line is the generation time, i.e. how long it takes for a population of cells to duplicate. For this purpose, 1×10^5 cells were seeded in five 25 cm^2 flasks and left to adhere for 24 hours at 37°C in a 5% CO_2 atmosphere. In the following two days the flasks were trypsinized and cells were counted using 50 µL of cell suspension added to 50 µL of 0.4% trypan blue vital dye (1:1). The number of cells in the total volume was calculated using equation 1. The results were plotted in a graphic (cell number versus time), from which the generation time was calculated using linear regression analysis.

3.2.3.2 *In vitro* cytotoxicity assays

For the elaboration of cytotoxicity assays, a series of compounds were chosen, including novel compounds obtained by chemical synthesis with potential interest for topical application: 1,2-dihydroxyxanthone (1,2-DHX), Ascorbic acid persulfate (ASS), Chlorogenic acid persulfate (ACS) and Resveratrol Glicoside Sulfate (RGS) synthesized in the Pharmaceutical Chemistry Laboratory, FFUP. Resveratrol (RSV), Resveratrol Glicoside (RSV-Gli) and *C. sativa* leaf extract were also characterized. RSV loaded nano and micro particles were also analyzed in this work. The concentrations tested and solvents are described in Table 6.

Table 6: Compounds and their concentrations tested in this work

Compounds	Solvents	Tested concentrations
1,2-DHX	DMSO	12.5*, 25, 50*, 100 and 200* µM
ASS	DMEM	100, 200, 350, 500 and 1000 µM
ACS	DMEM	100, 200, 350, 500 and 1000 µM
RGS	DMEM	100*, 350, 500*, 1000 and 5000* µM
RSV	DMSO	25*, 50, 100, 200*, 350 and 500* µM
RSV-Gli	DMSO	25*, 50, 100, 200*, 350 and 500* µM
C. sativa leaf extract	H_2O:Glicerin (1:1)	5*, 50, 100*, 250, 500* µg/mL
Nano and micro particles	DMEM	0.01*, 0.05* and 0.1* % (m/v)

* concentrations tested in Trypan Blue exclusion assay

To assess the cytotoxic effect of the selected compounds on HaCaT cells, five cytotoxicity assays were performed after an initial optimization step. For all compounds, except the ASS and ACS, cytotoxicity assays were conducted to assess metabolic activity (MTT and AlamarBlue® assays), to evaluate the lysosomal integrity (Neutral Red assay) and to assess plasma membrane integrity (Trypan Blue and Propidium Iodide assay). For ASS and ACS only MTT reduction assay was realized due to the low amount compound available.

All experiments were repeated at least three times. All results from the cytotoxicity assays were expressed in percentage of cell viability relatively to positive control (cells treated with the solvent) and when applied the concentration able to reduce cell viability by 50% (IC_{50}) was calculated using linear regression analysis.

3.2.3.2.1 MTT reduction assay

The MTT is a water soluble tetrazolium salt which is reduced to formazan (precipitated of violet color) only by metabolically active cells. This assay was described with more detail on 1.2.2.2.

- Optimization of the MTT reduction assay conditions

For MTT reduction assay, the optimal cell density was previously selected. For this end, cell densities of 2.5×10^3, 5×10^3, 1×10^4, 2×10^4 and 5×10^4 cells/well were seeded on 96 well tissue culture plates (150 µL/well) and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. The next day, the medium was removed, cells were washed one time with DPBS, and fresh culture medium was added and cells were incubated at 37°C in a 5% CO_2 atmosphere for another 24 hours. On the third day, a 5 mg/mL MTT stock solution was prepared in DPBS and mixed by vortex until dissolved and then diluted 1:10 in serum free DMEM. This solution was protected from light and prepared on the day of use. The medium was removed, cells were washed one time with DPBS and 150 µL of serum free DMEM containing 0.5 mg/mL MTT was added. After 2 hours of incubation incubated at 37°C in a 5% CO_2 atmosphere the reagent was removed and followed by the addition of 150 µL DMSO to dissolve the formazan crystals. Finally, the absorbance of formazan was determined at 570 nm using a plate reader (Biotek Instruments, Synergy HT multi-mode microplate reader).

- Study of the cytotoxic effect of the tested compound's using MTT reduction assay

After optimization of the method, 1×10^4 cells/well (optimal cell density) were seeded and incubated at 37°C in a 5% CO_2 atmosphere. After 24 hours of incubation, the medium was removed, cells were washed one time with DPBS and different test compounds were added and incubated at 37°C in a 5% CO_2 atmosphere for another 24 hours. Ten percent DMSO was used as positive control. On the third day, the procedure was the same as described above.

The results were expressed as the absorbance ratio of treated to control cells:

$$\%\ \text{Cell Viability} = \frac{\text{As}}{\text{Ac}} \times 100 \quad \text{(equation 2)}$$

where As is the absorbance of treated cells and Ac the absorbance of control cells (untreated cells).

In order to choose the maximum concentration that does not induce cytotoxicity, DMSO was tested at different concentrations in this assay. Up to 1% had no influence on cell viability in the methods used and so the DMSO concentration did not exceed 1%.

3.2.3.2.2 AlamarBlue® reduction assay

The AlamarBlue® is an oxidized form of redox indicator that is blue in color and non-fluorescent. When incubated with viable cells, the reagent changes color from blue to pink and become fluorescent. This assay was described with more detail on 1.2.2.1.

- Optimization of AlamarBlue® reduction assay conditions

For AlamarBlue® reduction assay, the optimal cell density and incubation time with AlamarBlue® were previously optimized. For this purpose, cell densities of 5×10^3, 1×10^4, 2×10^4 and 4×10^4 cells/well were seeded on 96 well tissue culture plates (150 µL/well) and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. The next day, the medium was removed, cells were washed one time with DPBS, and fresh culture medium was added and cells were incubated 37°C in a 5% CO_2 atmosphere for another 24 hours. Ten percent AlamarBlue® of the volume in the well was added to each well and incubated at 37°C in a 5% CO_2 atmosphere. AlamarBlue® incubation times of 1, 2, 3 and 4 hours were tested. Finally, the absorbances at 570 nm (reduced form) and 600 nm (oxidized form) were determined using a plate reader.

The % reduction of AlamarBlue® was calculated using the following equation:

$$\%\ \text{Reduction of AlamarBlue®} = \frac{(\mathrm{E_{oxi}}600 \times \mathrm{A_{570}}) - (\mathrm{E_{oxi}}570 \times \mathrm{A_{600}})}{(\mathrm{E_{red}}570 \times \mathrm{C_{600}}) - (\mathrm{E_{red}}600 \times \mathrm{C_{570}})} \times 100 \quad \text{(equation 3)}$$

where:

$E_{oxi}570$ = molar extinction coefficient (E) of oxidized AlamarBlue® at 570nm = 80586

$E_{oxi}600$ = E of oxidized AlamarBlue® at 600nm = 117216

A_{570} = absorbance of test wells at 570nm

A_{600} = absorbance of test wells at 600nm

$E_{red}570$ = E of reduced AlamarBlue® at 570nm = 155677

$E_{red}600$ = E of reduced AlamarBlue® at 600nm = 14652

C_{570} = absorbance of negative control well (medium, AlamarBlue®, no cells) at 570nm

C_{600} = absorbance of negative control well (medium, AlamarBlue®, no cells) at 600nm

- Study of the cytotoxic effect of the tested compound's using AlamarBlue® reduction assay

After optimization of the method, 2×10^4 cells/well (optimal cell density) were seeded incubated at 37°C in a 5% CO_2 atmosphere. After 24 hours of incubation, the medium was removed, cells were washed one time with DPBS and the different test compounds were added and incubated at 37°C in a 5% CO_2 atmosphere for another 24 hours. Ten percent DMSO was used as positive control. Ten percent AlamarBlue® of the volume in the well was added to each well and incubated at 37°C for 4 hours (optimal incubation time). Finally, the absorbance at 570 nm and 600 nm was determined using a plate reader.

The % reduction of AlamarBlue® was calculated according to equation 3.

3.2.3.2.3 Neutral Red uptake assay

The amount of NR dye incorporated in the cells represents lysosomal functionality, as this dye easily penetrates viable cell membranes and accumulates intracellularly in lysosomes. This assay was described with more detail on 1.2.1.4.

- Optimization of NR uptake assay conditions

For NR uptake assay, the optimal cell density, the concentration of the NR solution and the incubation time with NR were previously optimized. For this purpose, 2.5×10^3, 5×10^3, 1×10^4, 2×10^4 and 5×10^4 cells/well were seeded on 96 tissue culture well plates (150 µL/well) and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. The next day, the medium was removed, cells were washed one time with DPBS, and fresh culture medium was added and incubated at 37°C in a 5% CO_2 atmosphere for another

24 hours. The medium was removed, cells were washed one time with DPBS and 150 µL of culture medium containing 33, 50, 100 and 330 µg/mL NR solution were added to each well and incubated at 37°C in a 5% CO_2 atmosphere. NR incubation times of 2, 3 and 4 hours were tested. NR tends to precipitate, so the medium with stain was incubated at 37°C in a 5% CO_2 atmosphere protected from light overnight, centrifuged at 1500 g for 10 minutes and filtered (5 µm) before use. After incubation time, NR solution was removed, cells were washed one time with DPBS at 37°C and 150 µL/well of the solution formed by 50% ethanol/1% acetic acid was added to extract the NR dye meanwhile captured within the cells. The plates were then placed in a microplate shaker for 10 minutes, at room temperature and protected from light. Finally, absorbance was measured at 540 nm.

- Study of the cytotoxic effect of the tested compound's using NR uptake assay

After optimization of the method, 2×10^4 cells/well (optimal cell density) were seeded and incubated at 37°C in a 5% CO_2 atmosphere. After 24 hours of incubation, the medium was removed, cells were washed one time DPBS and different test compounds were added and incubated at 37°C in a 5% CO_2 atmosphere for another 24 hours. Ten percent DMSO was used as positive control. The medium was removed, cells were washed one time with DPBS at 37°C and 150 µL of complete DMEM containing 50 µg/mL NR (optimal concentration) was added to each well and incubated for 3 hours (optimal incubation time) protected from light. After incubation time, NR solution was removed, cells were washed one time with DPBS and 150 µL/well of the solution formed by 50% ethanol/1% acetic acid was added to extract the NR dye meanwhile captured within the cells. The plates were then placed in a microplate shaker for 10 min, at room temperature and protected from light. Finally absorbance was measured at 540 nm.

The results were expressed as the absorbance ratio of treated to control cells, using Equation 2.

3.2.3.2.4 Trypan Blue exclusion assay

The trypan blue is a vital stain used to stain dead cells. This assay was described with more detail on 1.2.1.6.

- Optimization of Trypan Blue exclusion assay conditions

For Trypan Blue dye exclusion assay, the optimal cell density was previously selected. For this end, cell densities of 1×10^4, 2×10^4, 5×10^4, 1×10^5, 2×10^5 and 3.5×10^5 cells/well were seeded on 12 well culture plates (1 mL/well) and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. The next day, the medium was removed, cells were washed one time with DPBS, and fresh culture medium was added and cells were incubated at 37°C in a 5% CO_2 atmosphere for another 24h. Then the contents of the wells were transferred into conical tubes, wells were washed one time with DPBS and also transferred into conical tubes. Then, the cells were detached from the bottom of the wells by the action of trypsin after 5-12 minutes and were centrifuged at 416 g for 5 minutes at room temperature. Cells were counted using 50 µL of cell suspension added to 50 µL of 0.4% trypan blue vital dye (1:1).

- Study of the cytotoxic effect of the tested compound's using Trypan Blue exclusion assay

After optimization of the method, 1×10^5 cells/well (optimal cell density) were seeded and incubated at 37°C in a 5% CO_2 atmosphere. After 24 hours of incubation, the medium was removed, cells were washed one time DPBS and different test compounds were added and incubated at 37°C for another 24 hours. Ten percent DMSO and 15% ethanol 70º were tested as positive control. On the third day, the procedure was the same as described above.

The % cell viability was calculated according to:

$$\%\ \text{Cell viability} = \frac{\text{Viable cells}}{\text{Total number cells}} \times 100 \quad \text{(equation 4)}$$

3.2.3.2.5 Propidium Iodide exclusion assay

PI is a dye that does not pass through intact cell membranes. This assay was described with more detail on 1.2.1.5.

- Study of the cytotoxic effect of the tested compound's using Propidium Iodide exclusion assay

Using the cell density previously optimized for Trypan Blue dye exclusion assay, 1×10^5 cells/well were seeded and incubated at 37°C in a 5% CO_2 atmosphere. After 24 hours of incubation at 37°C in a 5% CO_2 atmosphere, the medium was removed, cells were washed one time DPBS and different test compounds were added and incubated at 37°C in a 5% CO_2 atmosphere for another 24 hours. Ten percent DMSO and 0.1% Triton X-100 were tested as positive control. On the third day, the contents of the wells were transferred into conical tubes, wells were washed one time with DPBS and also transferred into conical tubes. Then, the cells were detached from the bottom of the wells by the action of trypsin after 8 minutes (optimized incubation time), complete DMEM was added to stop the action of trypsin and the content was centrifuged at 416 g for 5 minutes at room temperature. The supernatant was discarded and the cells washed two times by addition of cold 5 mL of DPBS + 2% FBS, centrifuging at 416 g for 6 minutes at a temperature of 4°C. Then, the supernatant was aspirated and the cells were ressuspended in 100 µL of cold DPBS. Content was transferred to 2 mL tubes and, finally, stained with 5 µL of PI working solution [10 µL of stock solution (10 mg of PI + 10 mL of Milli Q water) in 1 mL of DPBS] 5 minutes before starting the analysis. Cells were acquired on the flow cytometer BD Accuri™ C6 and at least ten thousand events were analyzed on the basis of size, complexity and emission of orange fluorescence, by setting up instrument settings for FSC, SSC and FL-2 and by using stained and unstained cells. Data were analyzed by using the BD Accuri™ C6 software version 1.0.264.21.

3.2.4 Statistical analysis

The data is presented as mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis was carried out using One-way analysis

of variance (ANOVA) followed by the Dunnett *post hoc* test (comparison to negative control), after confirming normality and homogeneity of variance with Shapiro-Wilk and Levene tests, respectively. Comparisons between loaded and empty nanoparticles were performed with the Tukey *post hoc* test. Statistical significance was considered when $p < 0.05$.

All statistical analyses were performed using the SPSS software (v 23.0; IBM, Armonk, NY, USA) and graphs were generated by GraphPad Prism for Windows (version 6.0; GraphPad Software, Inc., USA).

4. RESULTS

4.1 Qualitative analysis of the phenolic composition of *C. sativa* leaf extract

Plant extracts are complex mixtures that include various compounds that may contribute to a particular biologic effect. Scavenging activity found for several reactive oxygen species by *C. sativa* EtOH:water (7:3) leaf extract and characterization of the phenolic compounds present in this extract have been documented previously [111].

HPLC analysis of the obtained extract was carried out to confirm the presence of these compounds and the results are shown in Figure 16.

Based on UV/Vis spectra and retention times of each compound and comparison with data obtained previously for this extract, it was possible to identify the main phenolic compounds of interest, such as: chlorogenic acid, hyperoside, rutin, isoquercitrin and ellagic acid. Hyperoside, isoquercitrin and rutin present co-elution, as a result of their structural affinity [113].

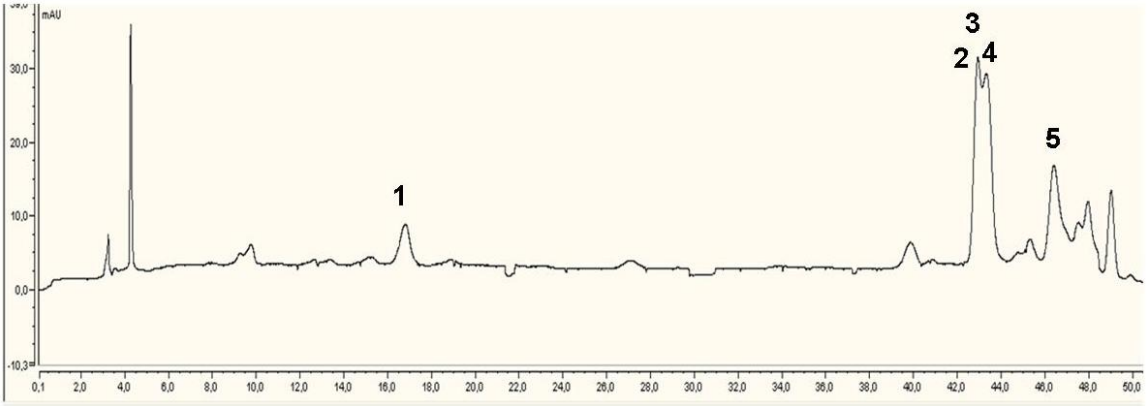

Figure 16: HPLC phenolic profile of *C. sativa* leaf extract. Detection at 350 nm. (1) chlorogenic acid; (2) hyperoside; (3) rutin; (4) isoquercitrin; and (5) ellagic acid. Based on [113].

4.2 Particle size measurements

The particle size was verified by measuring the particle size distribution using laser diffractometry. The mean size (Dv10, Dv50 and Dv90) of the lipid nanoparticles and polymeric microparticles, are presented in Table 7 and Table 8, respectively. Span refers to the bandwidth of the size distribution and it is determined by the following equation:

$$\text{Span} = \frac{\text{Dv90} - \text{Dv10}}{\text{Dv50}} \quad \text{(equation 5)}$$

Table 7: Lipid nanoparticles size measurements after preparation. The results are expressed as mean ± SD of different batches (n=14-21)

	Dv10 Mean ± SD [nm]	Dv50 Mean ± SD [nm]	Dv90 Mean ± SD [nm]	Span Mean ± SD
SLN empty	124 ± 23	283 ± 39	720 ± 99	2.165 ± 0.570
SLN-RSV	134 ± 26	296 ± 53	707 ± 149	1.971 ± 0.543
NLC empty	160 ± 19	369 ± 61	1074 ± 459	2.374 ± 0.719
NLC-RSV	140 ± 16	305 ± 40	746 ± 247	1.938 ± 0.450

Table 8: Polymeric microparticles size measurements after preparation. The results are expressed as mean ± SD of different batches (n=2)

	Dv10 Mean ± SD [µm]	Dv50 Mean ± SD [µm]	Dv90 Mean ± SD [µm]	Span Mean ± SD
Poly empty	0.691 ± 0.035	3.930 ± 0.651	15.650 ± 3.889	3.775 ± 0.352
Poly-RSV	2.070 ± 0.933	5.585 ± 1.860	14.500 ± 0.424	2.378 ± 0.882

4.3 Characterization of the HaCaT cell line

In Figure 17 depicts the results obtained for the determination of doubling time of HaCaT cells.

The calculated generation time was 23.82 hours. In the work by Rekus, of the generation time in HaCaT cells was approximately 26 hours [116].

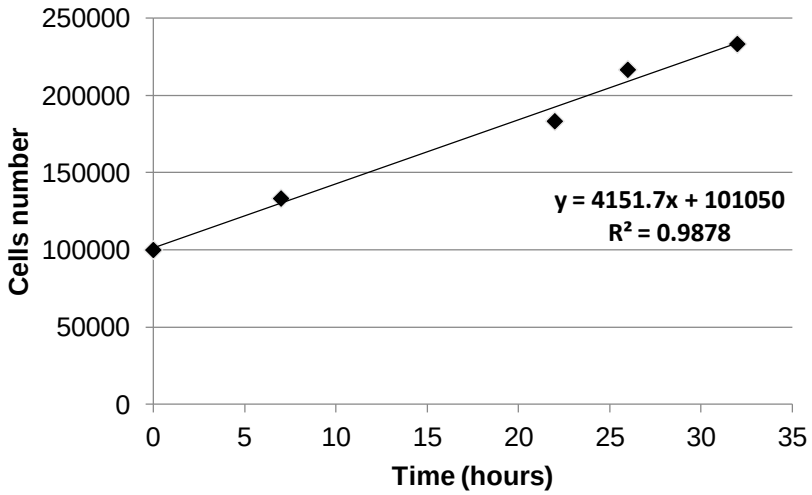

Figure 17: Determination of HaCaT cells doubling time by linear regression analysis.

4.4 *In vitro* cytotoxicity assays

The main objective of this work was to evaluate the cytotoxic effect of several compounds with putative interest for topical application. For this purpose, MTT reduction, AlamarBlue® reduction, NR uptake, Trypan Blue exclusion and Propidium iodide exclusion *in vitro* assays were used.

4.4.1 MTT reduction assay

The yellow tetrazolium MTT is reduced by metabolically active cells. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means and is thus directly proportional to the cell viability.

- Optimization of the MTT reduction assay conditions

Before assessing the cytotoxic effect of the selected compounds with the MTT reduction assay, different cell densities of HaCaT cells were tested in order to choose the optimal cell density. Through the analysis of Figure 18, the density of 1×10^5 cells/well was chosen because it provided an absorbance lower than 1.0 AU (0.797 AU) and within the linear range. This also contributes to a proper sensitivity of the method of assessment of cytotoxicity.

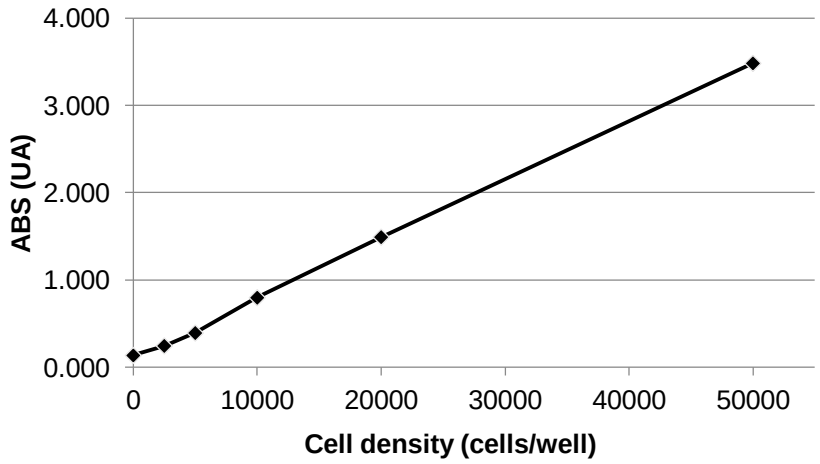

Figure 18: Evaluation of the linearity of the MTT reduction assay in function of HaCaT cell density.

- Study of the cytotoxic effect of the tested compound's using MTT reduction assay

After choosing the optimal cell density for MTT reduction assay, the selected compounds were tested. Figure 19 shows the results of the viability assay for RSV and its derivatives. A significant decrease in cell viability occurred for cells exposed from the concentration of 100 µM for RSV (A) and at 5000 µM for RGS (C) when

compared to cells treated with the solvent (negative control). RSV-Gli (B) did not induce significant alterations of HaCaT cells viability.

For the positive control (10% DMSO) HaCaT cells showed a cell viability of 12.67 ± 4.21%.

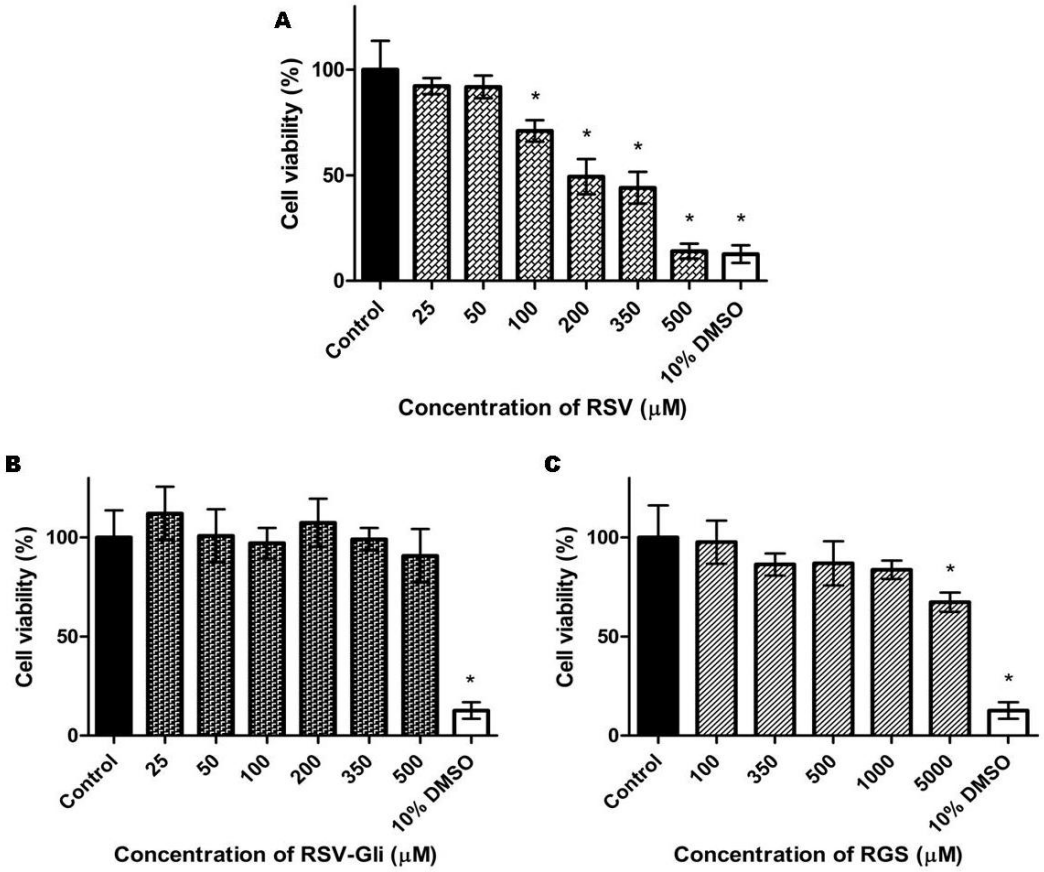

Figure 19: Cell viability of HaCaT cell line exposed to (A) RSV, (B) RSV-Gli and (C) RGS, determined by the MTT reduction assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

Figure 20 shows the results of the viability assay for lipid nanoparticles. Both the loaded and the empty nanoparticles in all tested concentrations showed a significant decrease in cell viability. A concentration-dependent decrease is observed mainly for SLN particles.

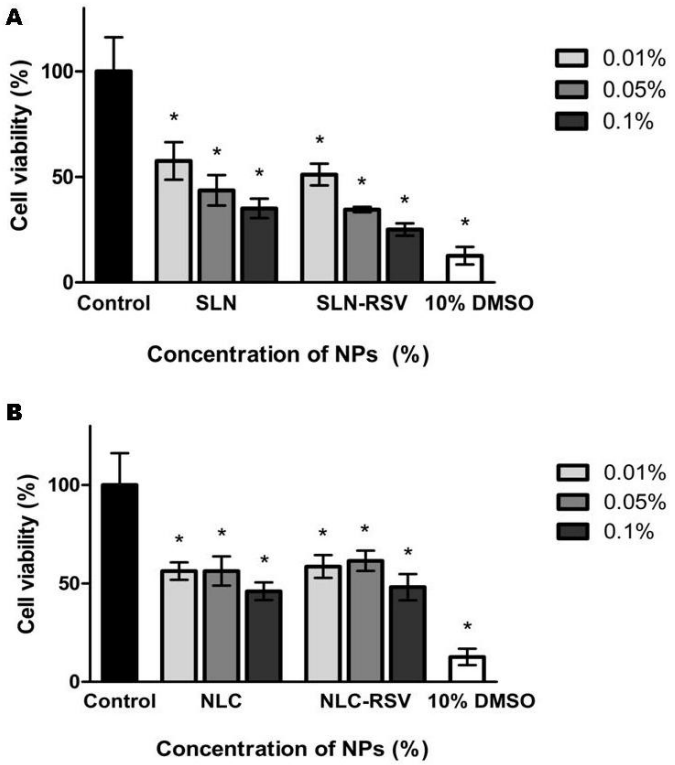

Figure 20: Cell viability of HaCaT cell line exposed to (A) SLN empty and SLN-RSV and (B) NLC empty and NLC-RSV, determined by the MTT reduction assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value. Comparisons between loaded and empty nanoparticles were performed with the Tukey post hoc test. ** P<0.05 data are statistically different.

In addition to the lipid nanoparticles, polymeric microparticles were also tested with the MTT reduction assay. However, the deposition of the microparticles on the surface of the cell monolayer and microparticle aggregation (as shown in Figure 21) led to a great variability of the results. For this reason, these particles were not included on subsequent cytotoxicity tests.

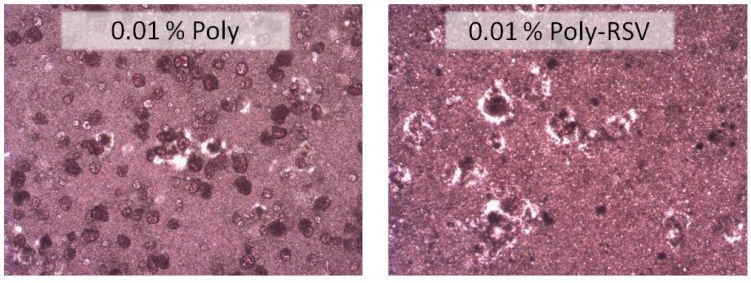

Figure 21: Observation under inverted microscope of HaCaT cell line with 0.01% polymeric microparticles after 24 hours incubation (10X magnification).

Figure 22 and 23 show the cell viability results for two of the compounds obtained by chemical synthesis, ASS and ACS, respectively. ASS caused significant cytotoxicity for all tested concentrations when compared to negative control, being the highest concentration tested (5000 µM) able to decrease the levels of viability to 62.49 ± 6.24%. A concentration-dependent effect was not found. For ACS, a significant decrease in cell viability has been detected from the concentration of 500 µM when compared to the negative control. For this compound, a concentration-dependent decrease of cell viability was observed.

As already mentioned, these two compounds were not tested with the remaining cytotoxicity assays due to the small amount available.

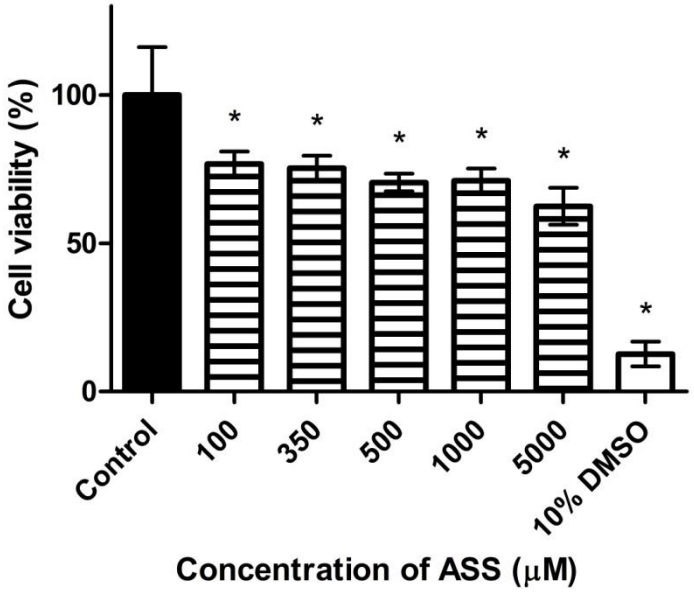

Figure 22: Cell viability of HaCaT cell line exposed to ASS, determined by the MTT reduction assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

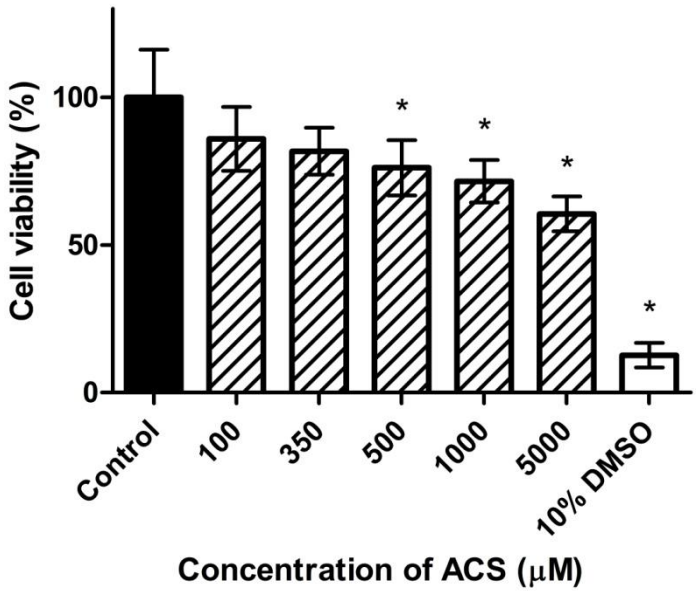

Figure 23: Cell viability of HaCaT cell line exposed to ACS, determined by the MTT reduction assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

Figure 24 shows the cell viability results obtained for 1,2-DHX. After the 24h incubation with HaCaT cells, 1,2-DHX caused a significant decrease in cell viability from the concentration of 100 µM. The cell viability was 36.14 ± 7.44% for HaCaT cells exposed to the concentration of 200 µM.

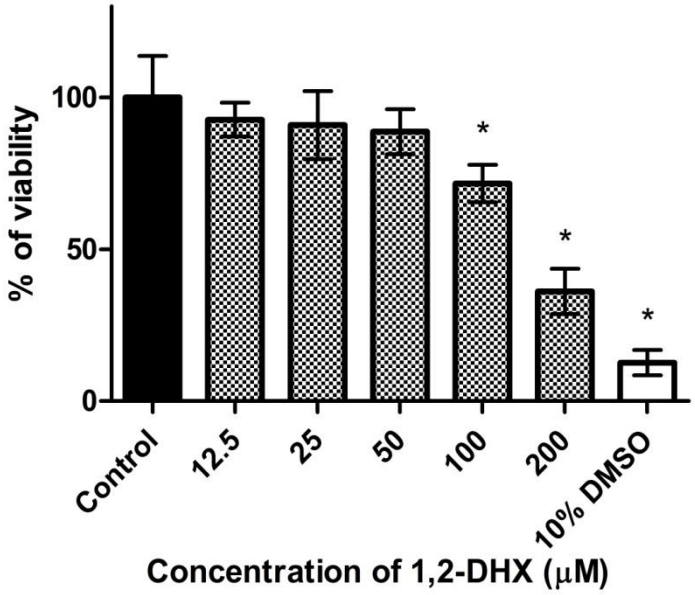

Figure 24: Cell viability of HaCaT cell line exposed to 1,2-DHX, determined by the MTT reduction assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

The ability of *C.sativa* EtOH:water (7:3) leaf extract to interfere with cell viability of the HaCaT cells after 24 hours incubation was evaluated. The Figure 25 shows that *C.sativa* extract caused significant cytotoxicity when compared to negative control only at the concentration of 500 µg/mL.

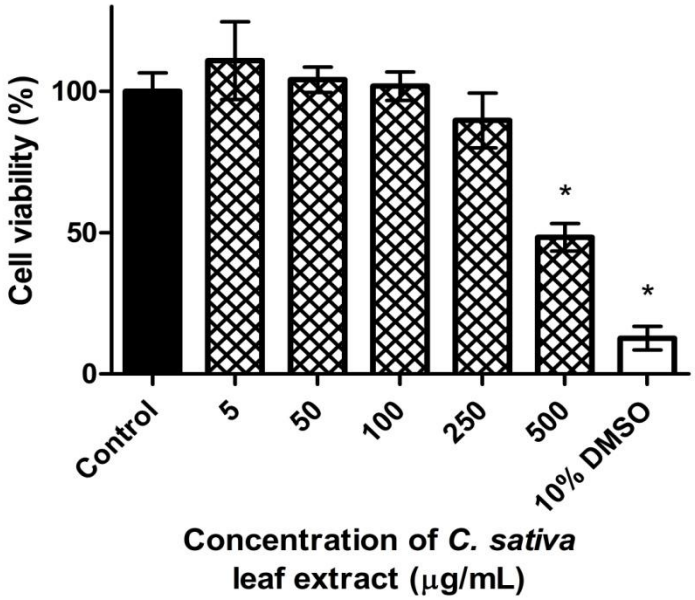

Figure 25: Cell viability of HaCaT cell line exposed to *C.sativa* leaf extract, determined by the MTT reduction assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

4.4.2 AlamarBlue® reduction assay

The AlamarBlue® reagent has as active ingredient rezasurin, a cell permeable compound that is blue in color and virtually non-fluorescent. Upon entering cells, resazurin is reduced to resorufin, a compound that is pink in color and highly fluorescent.

- Optimization of the AlamarBlue® reduction assay conditions

Before assessing the cytotoxic effect of the selected compounds with the AlamarBlue® reduction assay, different cell densities of HaCaT cells and different incubation times with AlamarBlue® were tested. Figure 26 shows the results obtained on this optimization step.

The reduction of AlamarBlue® increased with increasing cell density up to the density of 2×10^5 cells/well, from this value, a plateau is reached. The reduction of AlamarBlue® also increased with increasing incubation time. Given all the results, the chosen test conditions were: cell density of 2×10^5 cells/well and incubation time of 4 hours.

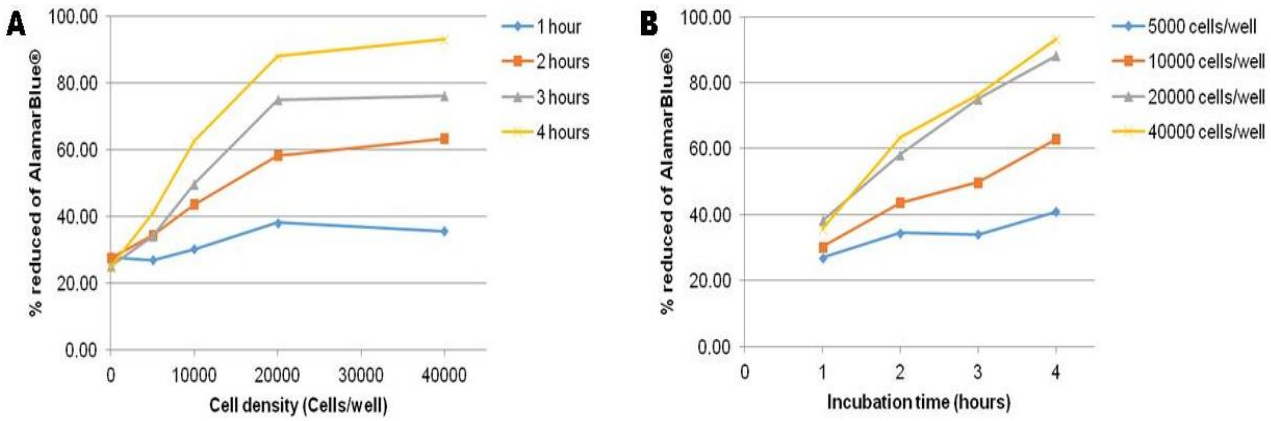

Figure 26: Graph of % reduced of AlamarBlue® obtained for HaCaT cell line. (A) % reduction of AlamarBlue® in function of the cell density and (B) % reduction of AlamarBlue® in function of the incubation time with AlamarBlue®.

- Study of the cytotoxic effect of the tested compound's using AlamarBlue® reduction assay

After choosing the optimal cell density and incubation time for AlamarBlue® reduction assay, the selected compounds were tested. Figure 27 shows the results of viability assay for RSV and its derivatives. A concentration-dependent decrease in cell viability was observed for RSV (A). A significant decrease in viability of cells occurred after exposure to RSV concentrations of 350 and 500 μM (A). For RSV-Gli (B) and RGS (C) no significant alterations of HaCaT cell viability were detected.

HaCaT cells incubated with positive control showed a cell viability of the 7.56 ± 6.29%.

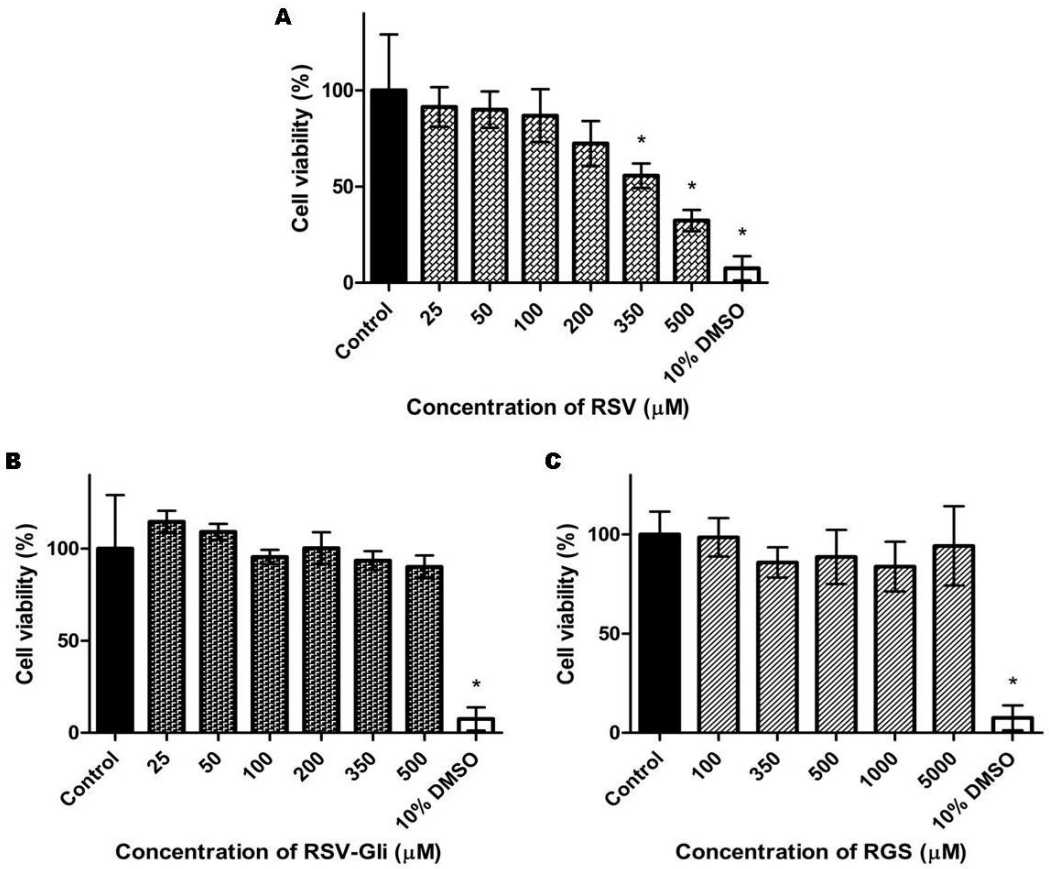

Figure 27: Cell viability of HaCaT cell line exposed to (A) RSV, (B) RSV-Gli and (C) RGS, determined by the AlamarBlue® reduction assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

Figure 28 shows the results of HaCaT cells exposed 24 hours with the lipid nanoparticles. The concentrations 0.1% SLN empty, 0.05% and 0.1% SLN-RSV showed a significant decrease in cell viability. In turn, 0.01% SLN increases the cell viability, under present experimental conditions. Empty and drug-loaded NLC did not induce significant alterations in HaCaT cell viability.

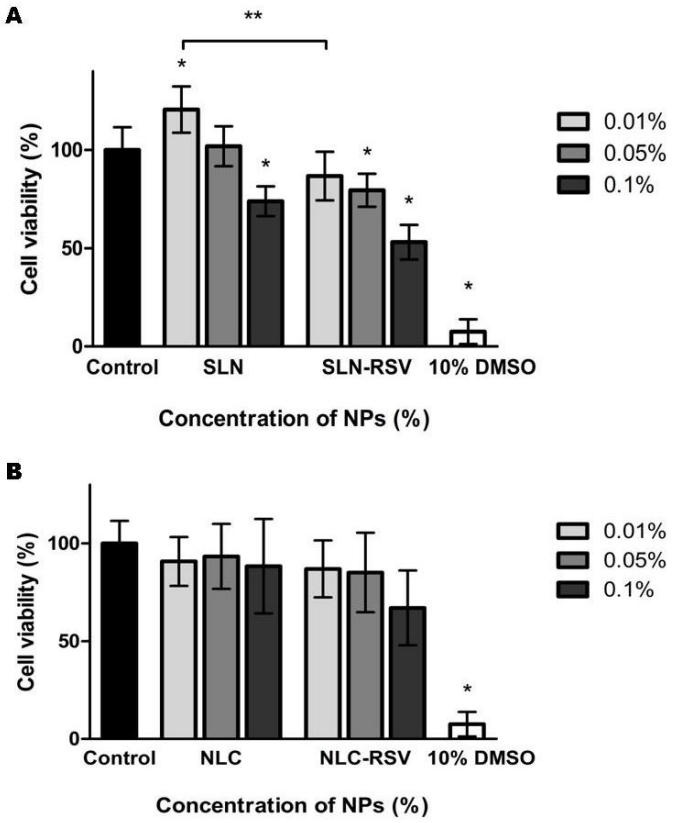

Figure 28: Cell viability of HaCaT cell line exposed to (A) SLN empty and SLN-RSV and (B) NLC empty and NLC-RSV, determined by the AlamarBlue® reduction assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value. Comparisons between loaded and empty nanoparticles were performed with the Tukey post hoc test. ** P<0.05 data are statistically different.

One of the distinct features of the experimental protocol of this assay is the absence of a washing step before measuring the absorbance. This results in the contribution of the optical density properties of the tested compounds to the measured absorbance. This was particularly relevant for lipid nanoparticles since they are dispersed in cell medium and for the higher concentrations, absorbance results higher than 1 UA were found.

Figure 29 shows the cell viability results obtained for 1,2-DHX. This xanthone caused a significant decrease in cell viability only at the concentration of 200 μM. The

cell viability was 54.51 ± 13.53% for HaCaT cells exposed to the concentration of 200 µM.

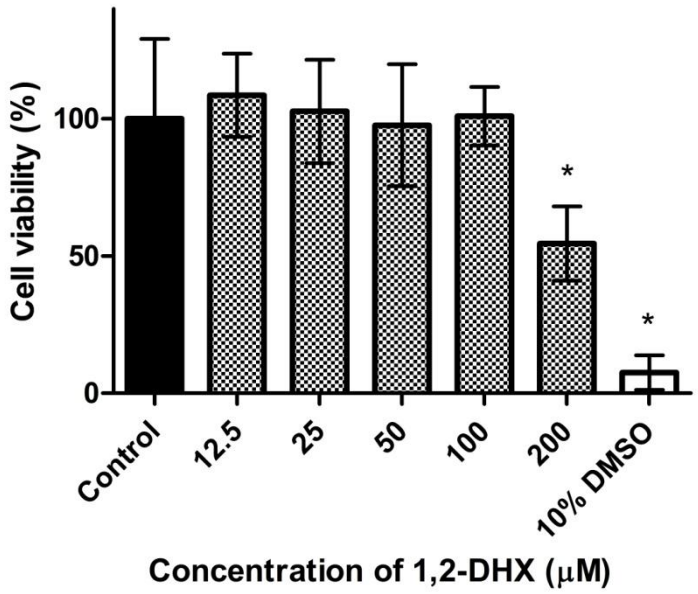

Figure 29: Cell viability of HaCaT cell line exposed to 1,2-DHX, determined by the AlamarBlue® reduction assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

The Figure 30 shows the results obtained for extract plant. In general, *C. sativa* leaf extract did not induce significant alterations of HaCaT cells, when available with AlamarBlue® reduction assay.

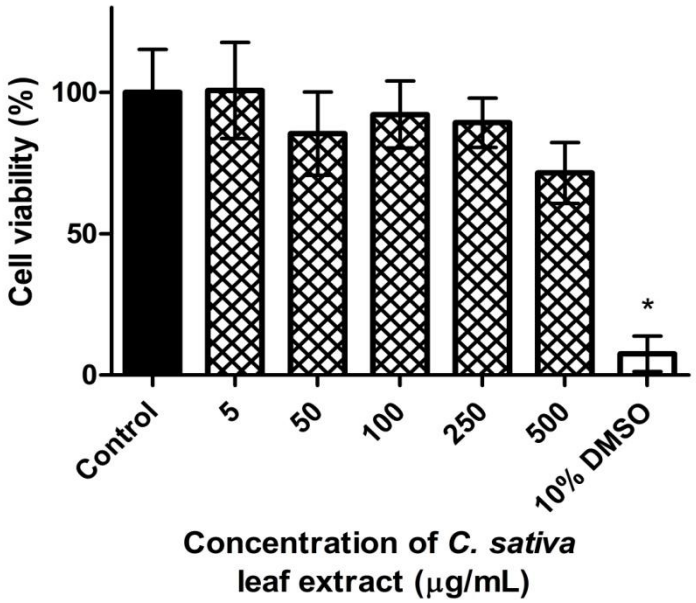

Figure 30: Cell viability of HaCaT cell line exposed to *C.sativa* leaf extract, determined by the AlamarBlue® reduction assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

4.4.3 Neutral Red uptake assay

The NR uptake assay was used to assess the integrity of the lysosomal membrane. Viable cells take up the dye by passive transport and incorporate the dye into lysosomes, whereas non-viable cells do not take up the dye. Thus, a decrease in NR cell content indicates damage of the lysosome and suggests a decrease in cell viability.

- Optimization of the NR uptake assay conditions

Before assessing the cytotoxic effect of the selected compounds with the NR uptake assay, different cell densities of HaCaT cells, concentrations of the NR solution and incubation times with NR solution were tested.

Selection of the appropriate final concentration of NR solution was based on the observation under inverted microscope of the different concentrations tested. For concentrations of 100 and 330 µg/mL the formation of crystals was observed, unlike concentrations of 33 and 50 µg/mL, as shown in Figure 31.

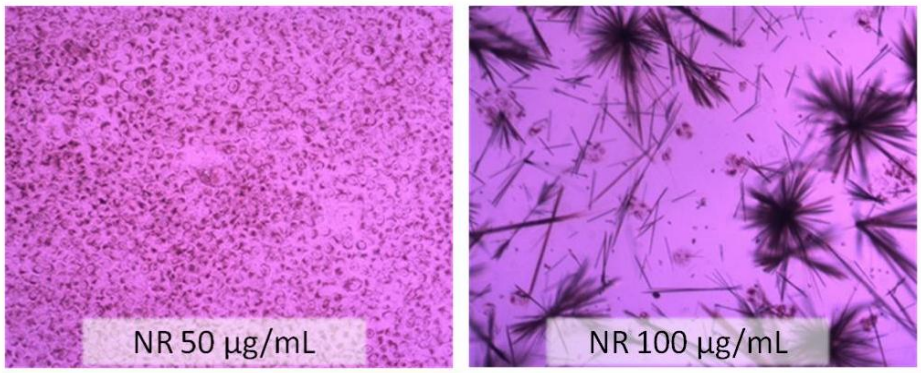

Figure 31: Observation under inverted microscope of HaCaT cell line in different tested concentrations the NR solution (10X magnification).

Figure 32 depicts the results obtained for the different cell densities and incubation times tested.

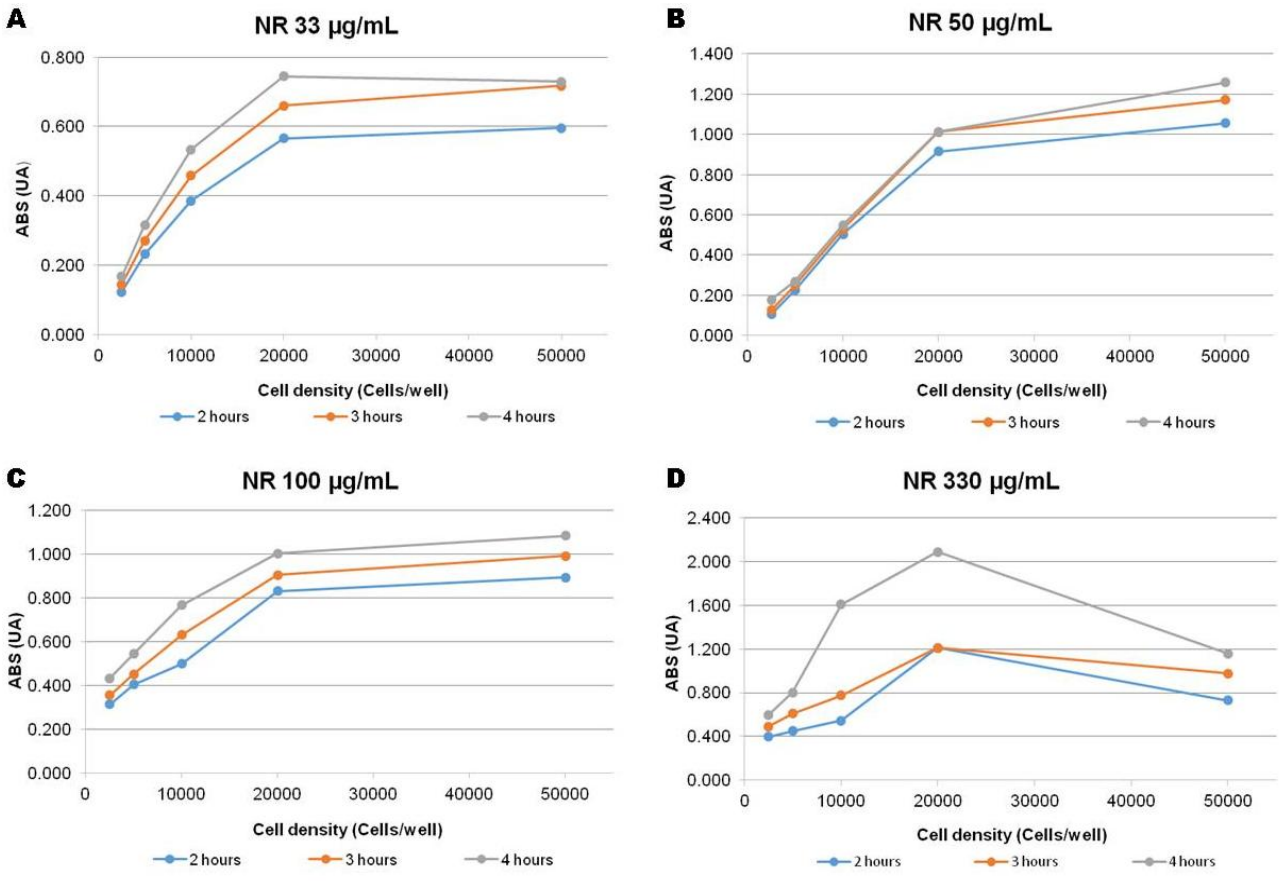

Figure 32: Graph of absorbance values obtained for different tested cell density, final concentration of the Neutral Red and incubation time with NR solution. (A) NR 33 µg/mL (B) NR 50 µg/mL (C) NR 100 µg/mL and (D) NR 330 µg/mL.

Given all the results, the chosen test conditions were: density of 2×10^5 cells/well, NR solution concentration of 50 µg/mL and incubation time of 3 hours.

- Study of the cytotoxic effect of the tested compound's using NR uptake assay

After choosing the optimal cell density of HaCaT cells, concentration of the NR solution and optimal incubation time for this assay, the selected compounds were tested. Figure 33 shows the results of viability assays using RSV and its derivatives. RSV (A) at concentration of 25 µM already caused significant cytotoxicity when compared to control cells, being the highest concentration tested (500 µM) able to decrease the levels NR uptake to 35.67 ± 8.17%. For RSV-Gli (B) and RGS (C) no significant alterations of HaCaT cells viability were detected in the range of concentrations tested.

The positive control caused a viability cell of the 14.55 ± 2.24%.

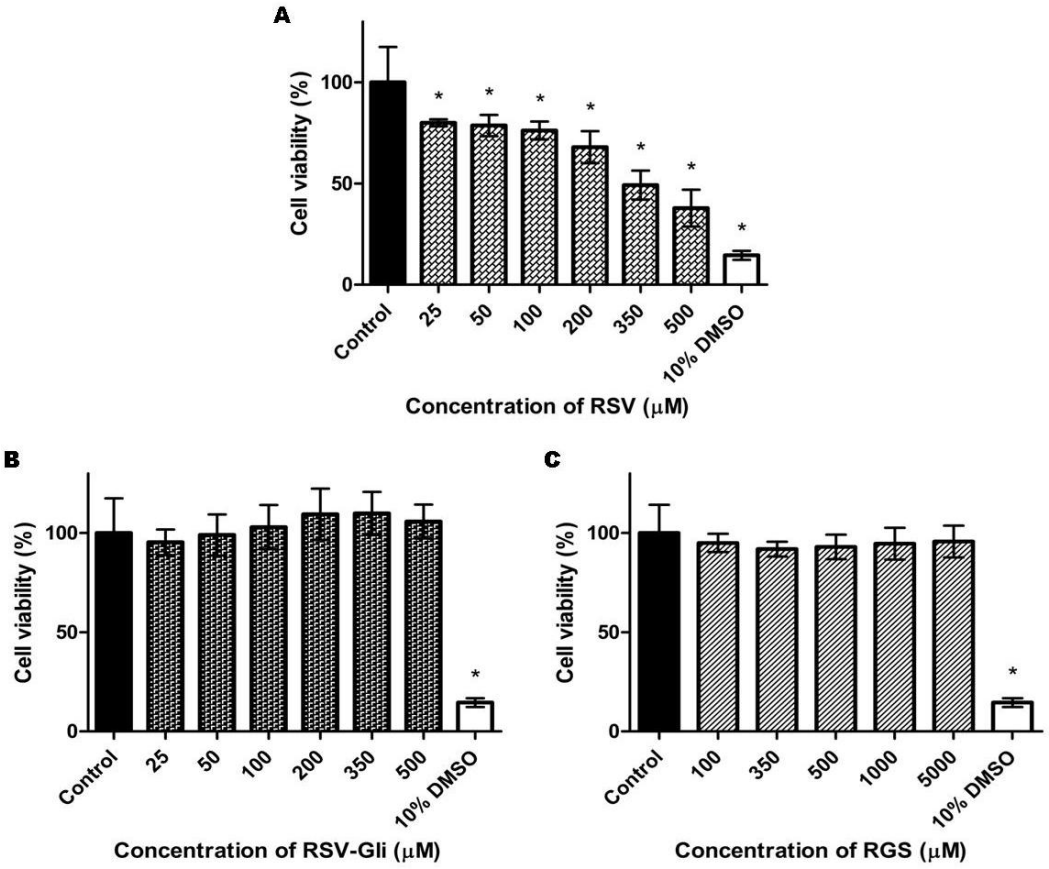

Figure 33: Cell viability of HaCaT cell line exposed to (A) RSV, (B) RSV-Gli and (C) RGS, determined by the NR uptake assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

Figure 34 shows the results of cell viability obtained with HaCaT cells exposed 24 hours with lipid nanoparticles. Regarding the SLN, there was a significant decrease in cell viability to 0.05% and 0.1% concentrations. For these lipid nanoparticles, a concentration-dependent decrease of cell viability was observed. Already the NLC showed significant decrease in cell viability for 0.1% NLC empty, 0.05% and 0.1% NLC-RSV. It is also important to underline the significant difference between the empty NLC and loaded-NLC with RSV for concentrations of 0.05% and 0.1%.

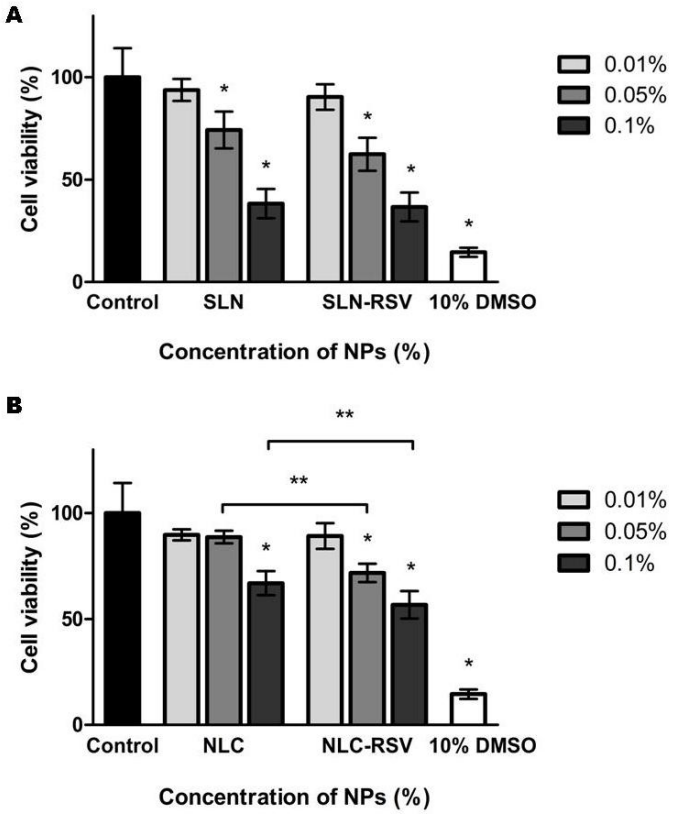

Figure 34: Cell viability of HaCaT cell line exposed to (A) SLN empty and SLN-RSV and (B) NLC empty and NLC-RSV, determined by the NR uptake assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * $P<0.05$ data are significantly different from the control value. Comparisons between loaded and empty nanoparticles were performed with the Tukey post hoc test. ** $P<0.05$ data are statistically different.

Figure 35 shows the cell viability results obtained for 1,2-DHX, after the 24h incubation with HaCaT cells. 1,2-DHX caused a significant decrease in cell viability from the concentration 50 µM when compared to negative control. For the highest concentration tested (200 µM) the cell viability was 41.96 ± 4.57%.

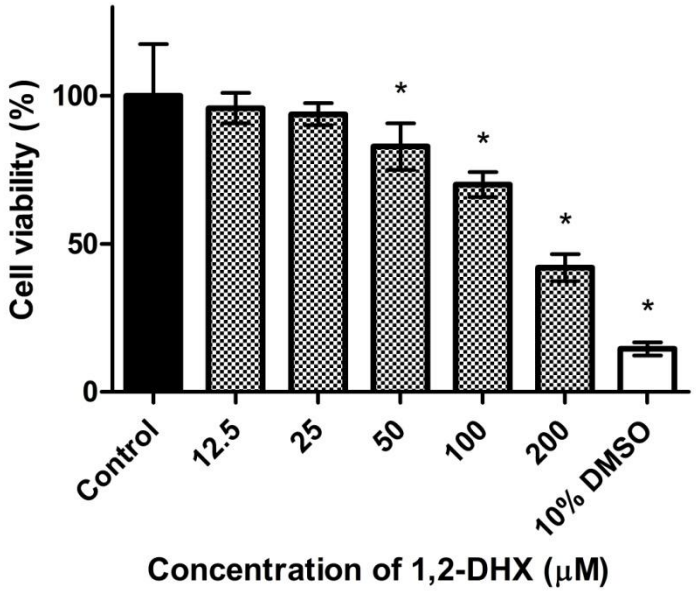

Figure 35: Cell viability of HaCaT cell line exposed to 1,2-DHX, determined by the NR uptake assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

Figure 36 shows the cell viability results obtained for plant extract utilized in this work. *C.sativa* leaf extract at concentrations of 250 and 500 µg/mL promoted a significant decrease in HaCaT cell viability in comparison to negative control. The viability was 56.13 ± 3.80% for cells exposed to concentration of 500 µg/mL.

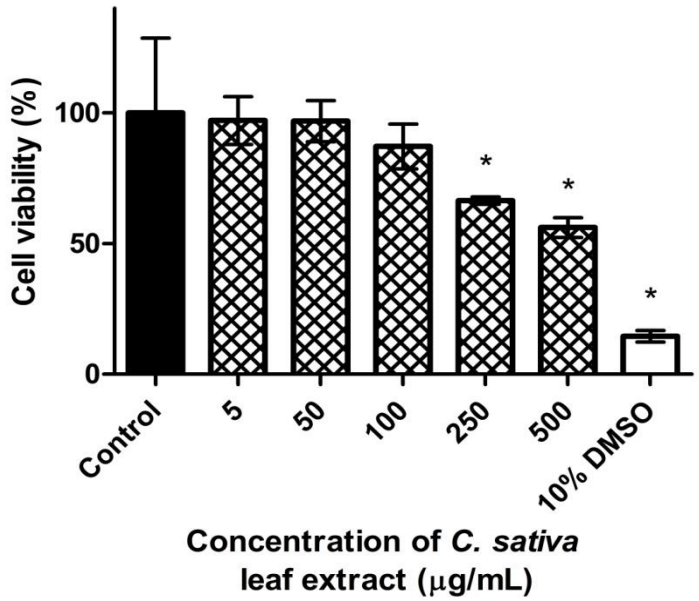

Figure 36: Cell viability of HaCaT cell line exposed to *C.sativa* leaft extract, determined by the NR uptake assay. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

4.4.4 Trypan Blue exclusion assay

The Trypan Blue exclusion assay was used to assess the integrity of the plasma membrane. This assay is based on the principle that the intact plasma membranes of live cells exclude trypan blue, whereas the dead cells do not.

- Optimization of the Trypan Blue exclusion assay conditions

Before assessing the cytotoxic effect of the selected compounds with the Trypan Blue exclusion assay, different cell densities of HaCaT cells were tested in order to choose the optimal cell density. Selection of the appropriate cell density for this assay was based on the observation under inverted microscope of the different cell densities tested.

Through the analysis of the Figure 37, with regard to confluence obtained for each cell density, 1×10^5 cells/well was chosen.

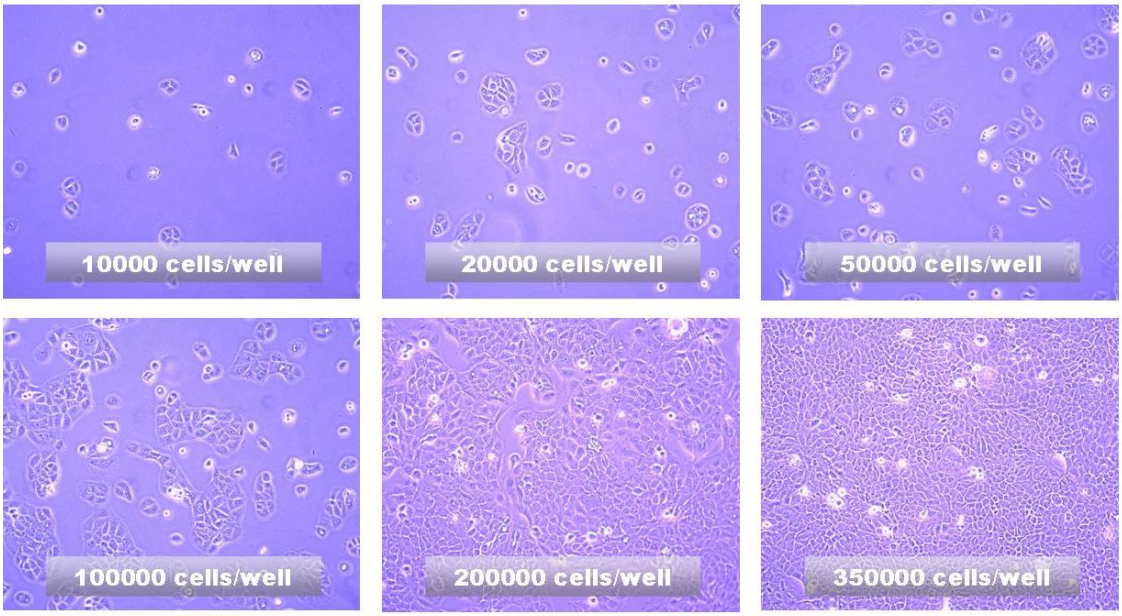

Figure 37: Observation under inverted microscope of HaCaT cell line in different tested cell densities, after 48 hours incubation (10X magnification).

Beyond the choice of the appropriate cell density, it was also necessary to test the positive control. While in the above cytotoxicity assays 10% DMSO proved to be a good positive control, for trypan blue reduction assay this was not verified. With 10% DMSO the cells detached and fragmented, which made it impossible to count them (Figure 38). In addition, 15% ethanol 70º was also tested, but only dead cells were counted and the number of total cells was reduced drastically, so it was decided not to include a positive control in our results.

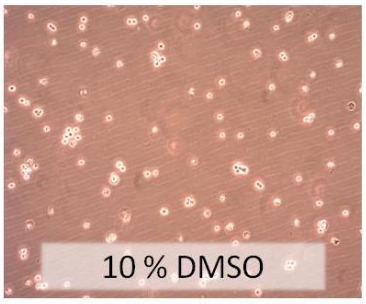

Figure 38: Observation under inverted microscope of HaCaT cell line with 10% DMSO after 24 hours incubation (10X magnification).

- Study of the cytotoxic effect of the tested compound's using Trypan Blue exclusion assay

After choosing the optimal cell density for trypan blue exclusion assay, the selected compounds were tested. Figure 39 shows the results of the viability assay for RSV and its derivatives. A significant decrease in cell viability occurred for HaCaT cells exposed from the concentration 200 µM for RSV (A) and at 5000 µM for RGS (C). RSV-Gli (B) did not induce significant alterations of HaCaT cells viability.

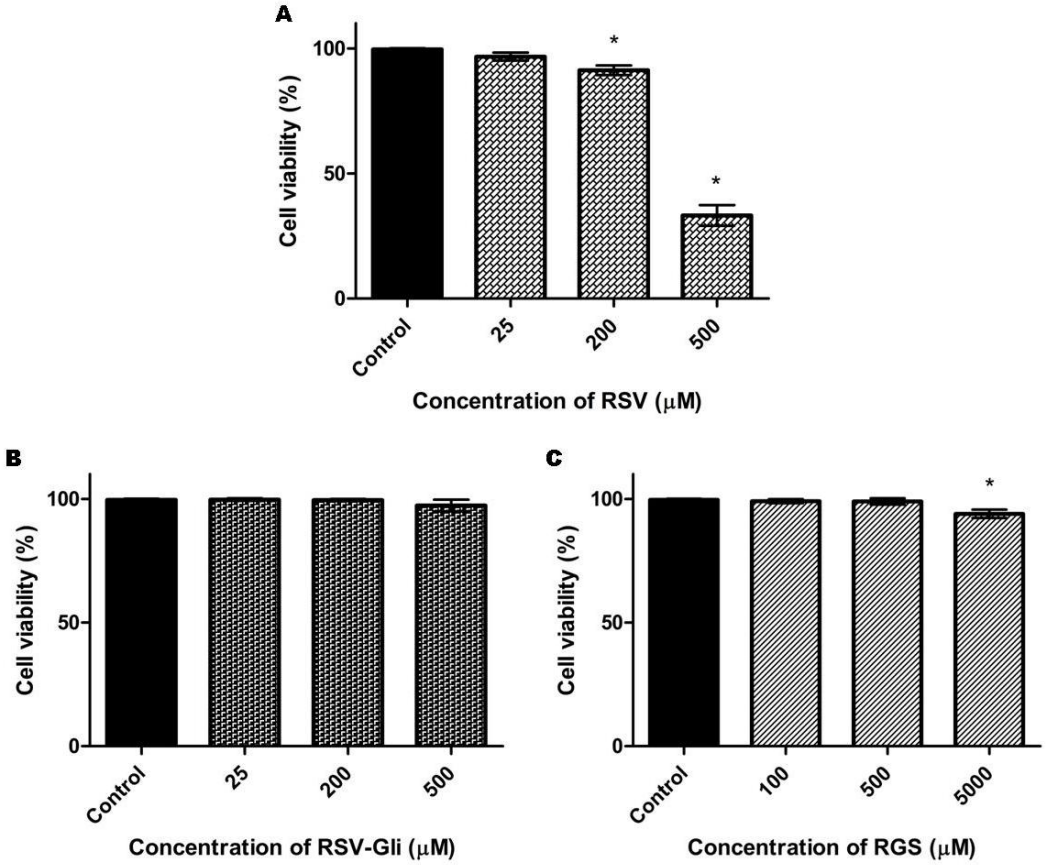

Figure 39: Cell viability of HaCaT cell line exposed to (A) RSV, (B) RSV-Gli and (C) RGS, determined by the Trypan Blue exclusion. Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

Figure 40 show the results of cell viability of HaCaT cells after 24 hours incubation with lipid nanoparticles. Regarding the SLN, a significant decrease in cell viability to concentration 0.1% SLN empty, 0.05% and 0.1% SLN-RSV was observed. Already the NLC showed significant decrease in cell viability for concentration 0.1%.

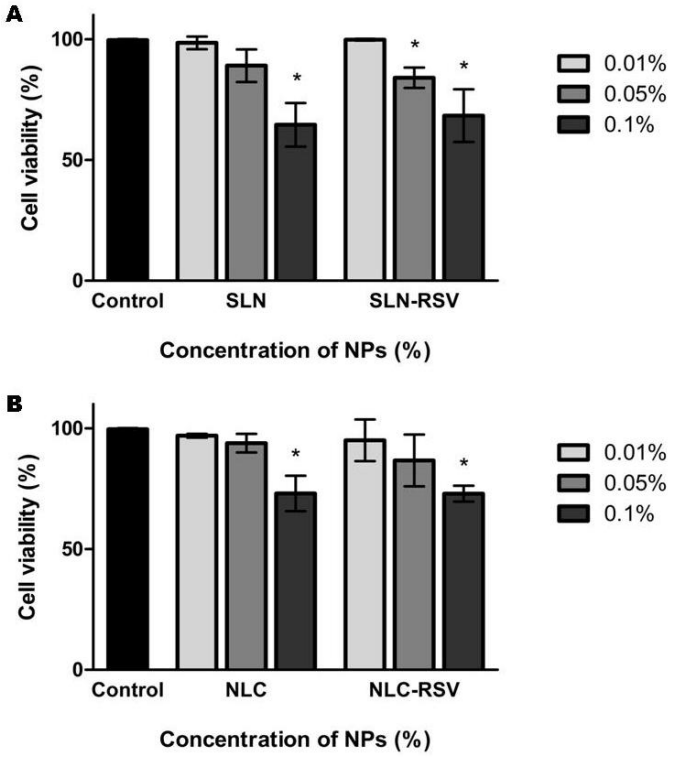

Figure 40: Cell viability of HaCaT cell line exposed to (A) SLN and SLN-RSV and (B) NLC and NLC-RSV, determined by the Trypan Blue exclusion assay. Data is presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value. Comparisons between loaded and empty nanoparticles were performed with the Tukey post hoc test. ** P<0.05 data are statistically different.

Results of the 1,2-DHX after 24 hours of incubation with HaCaT cells are shown in Figure 41. A significant decrease in the cell viability is detected with 200 µM concentration. For this concentration the cell viability was 74.29 ± 12.54%.

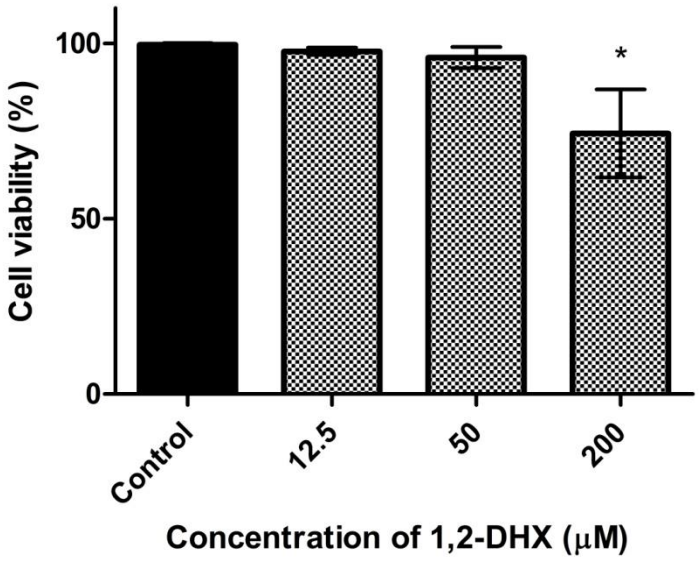

Figure 41: Cell viability of HaCaT cell line exposed to 1,2-DHX, determined by the Trypan Blue exclusion assay. Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

Results of cell viability of HaCaT cells after 24 hours incubation with *C.sativa* leaf extract are shown in Figure 42 and no significant alterations of cells viability were detected in the range of concentrations tested. There were some difficulties in evaluate the cytotoxic effects of this compound by Trypan blue exclusion assay. Indeed, after microscope analysis without the dye addition, dead cells were observed. Probably the solvent used, glycerin, may interfere with the density of the suspension leading to unsuitable centrifugation time and g-force that may damage the cells.

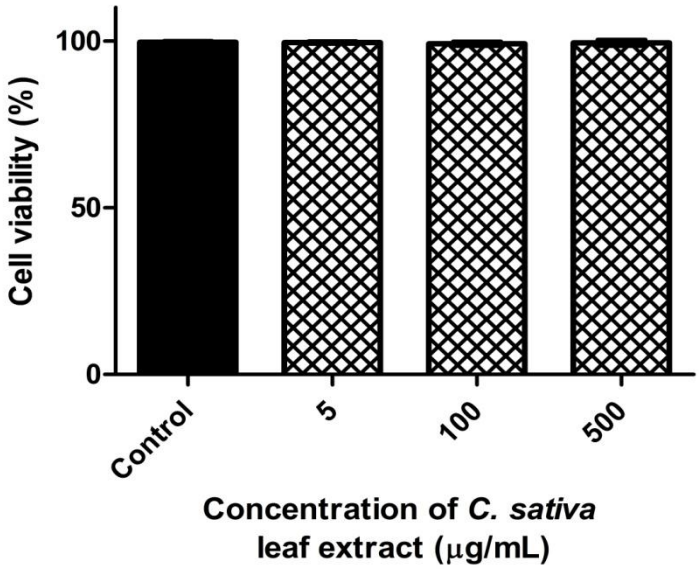

Figure 42: Cell viability of HaCaT cell line exposed to *C.sativa* leaf extract, determined by the Trypan Blue exclusion assay. Data are presented as mean ± SD (n=3-9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. * P<0.05 data are significantly different from the control value.

4.4.5 Propidium Iodide exclusion assay

Propidium iodide is a dye that does not pass through intact cell membranes, is generally excluded from viable cells.

Representative histograms obtained by flow cytometry for unstained cells and cells stained with PI are shown in Figure 43. The results showed that the negative control (untreated cells) presented a very high rate of PI incorporation probably due its high concentration. Optimization of several protocol conditions, including the PI and cell concentration, was needed and was not achieved due to time restrictions. Therefore we chose not to test the compounds with this methodology.

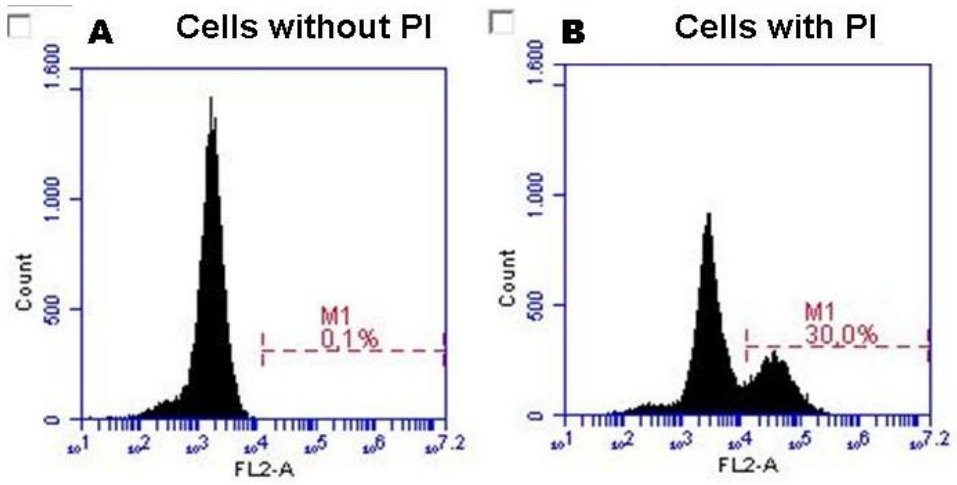

Figure 43: Representative histograms of unstained cells (A) PI stained cells (B) obtained by flow cytometry (BD ACCURI C6 software).

4.4.6 Comparison between the results of the different *in vitro* cytotoxicity assays

The IC_{50} values for cytotoxic effect and values of the lowest concentration that promote a significance statistically decrease to the initially cell viability, depending on the assay used are represented in Table 9.

For the chemicals and the plant extract tested on this work, MTT reduction and NR uptake assays presented higher sensitivity compared to the other assays. The toxicity was observed with NR uptake assays at lower concentrations, except for RGS and lower IC_{50} values were found with the MTT reduction assay. It should be noticed however that these assays were performed with different conditions such as different cell densities according to the results of the optimization step.

A wide variability was observed for all lipid nanoparticles which is a manifestation of the difficulties of analyzing their cytotoxicity *in vitro*.

Table 9: IC_{50} values and the values of the lowest concentration that promote a significance statistically decrease to the initially cell viability for different cytotoxicity assays used in HaCaT cell line

Cytotoxicity assays	MTT reduction		AlamarBlue® reduction		Neutral Red uptake		Trypan Blue exclusion	
	IC_{50} ± SD	*****	**IC_{50} ± SD**	*****	**IC_{50} ± SD**	*****	**IC_{50} ± SD**	*****
SLN empty	0.031 ± 0.021 %	0.01 %	> 0.1 %	0.01 %	0.084 ± 0.011 %	0.05 %	> 0.1 %	0.1 %
SLN-RSV	ND	0.01 %	> 0.1 %	0.05 %	0.077 ± 0.010 %	0.05 %	> 0.1 %	0.05 %
NLC empty	0.074 ± 0.020 %	0.01 %	> 0.1 %	-	> 0.1%	0.1 %	> 0.1 %	0.1 %
NLC-RSV	0.085 ± 0.005 %	0.01 %	> 0.1 %	-	> 0.1%	0.05 %	> 0.1 %	0.1 %
1,2-DHX	155.43 ± 8.15 µM	100 µM	> 200 µM	200 µM	172.40 ± 14.37 µM	50 µM	> 200 µM	200 µM
***C. sativa* leaf extract**	479.19 ± 14.90 µg/mL	500 µg/mL	> 500 µg/mL	-	> 500 µg/mL	250 µg/mL	> 500 µg/mL	-
RSV	174.47 ± 11.18 µM	100 µM	387.27 ± 6.88 µM	350 µM	383.19 ± 53.81 µM	25 µM	412.62 ± 14.79 µM	200 µM
RGS	> 5000 µM	5000 µM	> 5000 µM	-	> 5000 µM	-	> 5000 µM	5000 µM
RSV-Gli	> 500 µM	-	> 500 µM	-	> 500 µM	-	> 500 µM	-
ACS	> 5000 µM	500 µM						
ASS	> 5000 µM	100 µM						

***** is the lowest concentration that promote a significance statistically decrease to the initially cell viability.

ND: not defined

(**-**) indicates no statistically significance obtained.

5. DISCUSSION

In the present work, the cytotoxicity of a series of ingredients with potential interest for topical application was studied. This study was performed in HaCaT cells, an immortal non-cancerous human keratinocyte cell line. Keratinocytes are the predominant cells in the first layers of the skin, the epidermis. Considering the intended route of administration for these materials, using a human keratinocyte cell line for their preliminary safety evaluation is a logical choice.

Toxicological testing is a regulatory requirement for cosmetic and pharmaceutical products. For cosmetics, each ingredient included in the cosmetic product must undergo toxicological evaluation, such as, local toxicity and corrosivity evaluation (skin and eye irritation), skin sensitization, dermal/percutaneous absorption, among others. In the case of the pharmaceutical products, beyond toxicological requirements, other nonclinical studies include pharmacology studies (e.g. cardiovascular, central nervous, respiratory systems) are required.

To investigate the potential cytotoxic effects of a series of ingredients on HaCaT cells, different cytotoxicity assays were performed (MTT reduction, AlamarBlue® reduction, NR uptake and Trypan Blue exclusion). Cytotoxicity assays are widely used in preliminary investigation of the toxicity, since they provide an important advantage in identification of the cytotoxic compounds [83]. These assays allow determining whether a compound or extract contains significant quantities of biologically harmful chemicals, therefore are useful for screening as they serve to separate toxic from nontoxic materials, providing predictive evidence of compound safety [82]. Toxicity assays are essentials for a process of screening large chemical libraries easier, however the selection of one cytotoxicity assay over another can be troublesome due to the availability of several assays that rely on different physiological mechanisms and endpoints [117].

When comparing the four cytotoxicity assays employed in this work to assess a series of ingredients with potential interest for topical application, it is obvious that the results obtained are not in close agreement. This can be explained by the different mechanism assessed, except in case of the MTT and AlamarBlue® assays which both measure the metabolic activity. In turn, NR and trypan blue assess the lysosomal integrity and cell membrane damage, respectively. It has been previously reported that different cytotoxicity assays can give different results depending on the test agent used and the cytotoxicity assay employed [59]. Kim and co-workers showed no correlation

between MTT reduction and trypan Blue exclusion assays for the tested compounds [118].

In this work, NR and MTT assays were the most sensitive in detecting toxic effects compared to the other assays utilized since the toxicity was observed with NR assay at lower concentrations, except for RGS, and lower IC_{50} values were found with the MTT assay. These differences among cytotoxicity assays may suggest that the intracellular effects due to exposure to compounds occur before any permanent cell membrane damage occur. There are evidences in which these two assays were also more sensitive when compared with LDH leakage and protein assays [38]. MTT reduction assay also was more sensitive in a study performed in HaCaT cells [119]. However in this study, the cell concentration used to perform the MTT test was lower than the one used for the other tests, thus we cannot exclude the fact that the inferior IC50 values obtained for the MTT assay might be due to the cells being exposed to higher levels of the compounds. This evidence emphasizes the need to be careful when comparing different cytotoxicity tests. In the case of NR assay, this dye uptake by cells occurs through a process requiring energy, being sensitive to substances that interfere with cell membrane and lysosomal permeability as well as the process of energy-dependent endocytosis [120], therefore expectable to be one of the most sensitive test.

RSV a compound know as a strong antioxidant [121], anti-inflammatory [122] and a cardioprotective agent [123], showed in general a significant decrease in cell viability from the concentration of 100 µM, except in the NR reduction assay. In this assay, exposure to RSV at 25 µM already caused a significant decrease in cell viability comparing to control, which was not dependent on the concentration up to 100 µM. Noteworthy, cell viability found in such cases was around 80%, which can be considered non-cytotoxic [124]. A study performed in a primary keratinocyte line showed that RSV did not induce significant cytotoxicity up to 50 µM [125], which is consistent with the majority of our results. Other studies have revealed that RSV inhibits the proliferation of normal human keratinocytes *in vitro* [125, 126]. Although it was not the aim of our study, in the Trypan Blue exclusion assay it was also found that after exposure to RSV at 25 µM the number of viable cells was lower than the one found for the control, even if no decrease in cell viability was observed. This putative anti-proliferative effect could be a confounding factor in the analysis of cytotoxicity results, as is the case of NR reduction assay.

Two RSV derivatives were also studied in this work. RSV-Gli was the only compound studied in this work that showed no cytotoxicity for the tested concentrations (25-500 µM). The concentration range was limited by the maximum concentration of the solvent (DMSO) that doesn't affect cell viability (1%). In this case, it would be

interesting to extend the concentration range to detect toxic effects. For the RGS, a significant reduction of HaCaT viability was observed at the highest concentration tested (5000 µM) and was around 67% with MTT reduction assay.

With regard to the results obtained from cytotoxicity assays of lipid nanoparticles a greater variability was observed. In some cases significant differences were observed between RSV-loaded and empty nanoparticles which were not consistent across the assays. We can generally assume that the toxicity observed is due to the particles rather than RSV. Indeed, the theoretical calculation of the concentration of RSV present in the highest concentration tested (0.1%), based on drug loading analysis previously performed, was 36.5 µM. But when free RSV was tested up 100 µM no significant evidence of cytotoxicity was detected, except for the NR assay. This suggests that the excipients used in the matrix of lipid nanoparticles or their physico-chemical features (e.g. size and charge) could be responsible for the cell death. There is evidence that only the size of the nanoparticles is not sufficient to explain the cytotoxic effects [102]. It would be relevant to study the cytotoxicity of the ingredients alone to assess their contribution to the observed toxic effects. In the work by Ridolfi and co-workers, they showed that up to 0.5 mg/mL cetyl palmitate-based SLN caused a decrease in HaCaT cell viability [127]. The type, amount and interaction of the surfactant with the lipid core on nanoparticles also affect the performance of cells in culture [102]. Moreover there is evidence that the binding of surfactant to the nanoparticles surface decrease its availability to interact with cells and consequently decreases its toxicity [128]. So it is essential testing of the unprocessed starting material present in the same quantities and ratios as in the final formulation to clarify if the observed toxicity effects are result of that particular combination of materials *per se* or of the nanoparticles in question [102].

On the other hand, the lipid nanoparticles may form aggregates in cell culture media or suffer changes to its structure, for example desorption of surfactants [102]. Therefore it is important to take into account the stability of the nanoparticles in cell medium [21, 102]. Studies performed in our laboratory with the same lipid nanoparticles, showed the zeta potential varied between 27 and 40 mV, which accounts for a good colloidal stability in aqueous dispersion [129].

The majority of cell viability studies of SLN/NLC were performed by MTT assay [102]. The greater variability obtained in this study for lipid nanoparticles can also be explained by possible interactions between test reagents (e.g. MTT) and the nanoparticles, inducing false positives [21]. Furthermore, there is also evidence that SLN and NLC in the dispersion themselves absorb visible light [102]. To avoid the possibility of this lipid interaction with the assay, a washing step before adding the test

reagent was included in the protocol of cytotoxicity assays employed in this work, except for AlamarBlue® reduction assay. In this case, as is a water soluble dye, the blank measurement was included in this assay. But in blank for lipid nanoparticles contributed to optical density properties, which suggests that this cytotoxicity assay is not suitable for these nanoparticles.

Regarding the polymeric microparticles the MTT reduction assay was the the only test used, and the aggregation and deposition of the microparticles on the surface of the cell monolayer led to a great variability of the results.

Two of the compounds obtained by chemical synthesis, ACS and ASS were only tested with the MTT reduction assay, due to the reduced amount obtained in the synthesis process. ASS promoted a significant decrease in cell viability at the lowest concentration studied (100 µM). However this effect was not concentration-dependent, up to 1000 µM and cell viability was around 75%, which can be considered non-cytotoxic [124], so this reduction in cell viability could be due to other effects such as an antiproliferative activity. Elucidation of the cytotoxicity should be further ascertained with other assays. ACS showed significant cytotoxicity only at the concentration of 500 µM.

1,2-DHX, another compound obtained by chemical synthesis, showed significant cytotoxicity at concentrations of 50 µM and higher. In the work by Silva and co-workers 1,2-DHX was tested at several concentrations up to 50 µM with MTT reduction and Neutral Red uptake assays using Caco-2 cells. Silva *et al.* found no significant cytotoxicity within the tested concentration range [130].

In the case of the plant extract tested, no significant alterations of HaCaT cells viability were detected after incubation with concentrations lower than 250 µg/mL. In the work by Almeida and co-workers, this extract was tested with the MTT reduction assay, using HaCaT cells. Almeida *et al.* observed that the extract-induced cytotoxicity at concentrations equal to or higher than 50 µg/mL [111]. The dissimilarities found in both studies in terms of results could be explained by the different conditions used on the assay, such as different cell densities that restrains data comparison.

As for the positive control used (10% DMSO), this proved to be a good cytotoxic compound, except for Trypan Blue exclusion assay. Indeed, microscopic observation of the cells incubated with DMSO in the trypan blue test, indicates that probably this reagent significantly compromised the cell membrane integrity, leading to cell burst and consequently unable the trypan blue dye to be up taken by dead cells.

Regardless of the used assay, well defined protocols are needed to obtain meaningful results. In any cytotoxicity assays to be used, optimization must be carried out for every cell model [27]. Assays must be performed at a uniform temperature and

controlled pH to ensure reproducibility of all wells and across a plate. The optimum incubation time and cell density must be empirically determined [27]. All the tests used in this study for measure cytotoxicity of the different compounds were optimized. In particular, NR uptake and Trypan Blue exclusion assays showed more challenges for its proper implementation. NR assay was very dependent on temperature, forming crystals when there was a variation in temperature. Trypan blue is a manual test involving several cares, such as, collection of all cells (live and dead) from the well and the correct count of all cells is required. During assay optimization and development, microscopic observations are necessary for confirmation of cellular toxicity. Some of the observations include for example, cell detachment or presence of cell fragments [83]. This was particularly important for Trypan Blue exclusion assay, where it was found that 10% DMSO is not a good positive control.

6. CONCLUSIONS

The aim of this work was to study the cytotoxicity of several ingredients with potential interest for topical application. Cytotoxicity assays are widely used in the preliminary investigation of the toxicity. From the study carried out in this Master dissertation we were able to determine the concentration range for each compound that can be used in future assays to confirm the potentialities of these raw materials for application on the skin. The results obtained are also relevant for other toxicological studies that are mandatory to meet regulatory requirements.

The optimization of each cytotoxicity assay allowed the establishment of the most appropriate experimental conditions, considering the cell line used. The optimal cell density and incubation time with the test reagent were selected as follows: MTT reduction assay 1×10^4 cells/well and 2 h; AlamarBlue® reduction assay 2×10^4 cells/well and 4 h; NR uptake assay 2×10^4 cells/well and 4 h and Trypan Blue exclusion assay 1×10^5 cells/well. The concentration of the NR solution used was 50 µg/mL.

In the case of the RSV and its derivatives, they can be generally regarded as safe at concentrations up to 100 µM for RSV, 500 µM for RSV-Gli and 1000 µM for RGS. *C. sativa* leaf extract and 1,2-DHX can be safely used in future studies at concentrations up to 100 µg/mL and 50 µM, respectively. In turn, it would be necessary to complement the basic toxicity evaluation of ACS and ASS using more cytotoxicity studies. In particular, the absence of a concentration-dependent effect observed for ASS should be elucidated in future studies.

The lipid nanoparticles prepared and tested in this work were not considered as promising topical ingredients due to the marked *in vitro* toxicity presented. Our study also demonstrated that the nanoparticles present several challenges for toxicological testing. Improvement of the methodologies used should take into account the interaction between test reagents and nanoparticles and light dispersion or absorption effects that can interfere with the cytotoxicity assays. It would also be relevant to study the cytotoxicity of the ingredients alone to assess their contribution to the observed toxic effects.

Polymeric particles showed deposition on the surface of the cell monolayer and aggregation which led to a great variability of the results. Following these observations,

it would be more relevant to test the *in vitro* toxicity of these particles using for example, reconstructed human epidermis.

When comparing the cytotoxicity assays used, some differences were noted, being the MTT reduction and NR uptake assays the most sensitive tests. These remarks further reinforce the importance of using different *in vitro* cytotoxicity tests, which rely on different mechanisms of cell death.

Further toxicological tests should be carried out to meet regulatory requirements before considering the use of the tested compounds in cosmetic or pharmaceutical products.

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